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It is generally believed, from previous investigations (1–4). that naturally occurring sphingomyelins consist of choline-phosphoric acid esters of sphingosine in which one OH group is free and the amino group is combined with fatty acids (lignoceric, stearic, and palmitic).
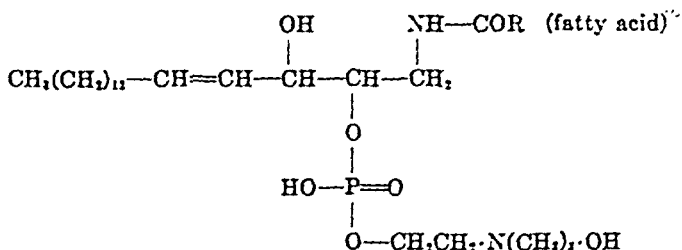
These lipids obtained from brain or other organs have been considered to be mixtures of palmitylsphingomyelin, stearylsphingomyelin, and lignocerylsphingomyelin, although the separation of these constituents has never been effected. However, lignocerylsphingosine is the only compound of fatty acid and sphingosine isolated from ether extracts of spleen (5) and lung (6). Such lipids, consisting of sphingosine linked to fatty acid through the acid amide (NH—CO) linkage, we have called ceramides (3, 5).

In the present investigation, it was hoped that by an enzymatic

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hydrolysis of sphingomyelin with a liver phosphatase the choline-phosphoric acid would be split from the three supposed ceramides, and the identification of these latter compounds would verify the structure of sphingomyelin suggested by Levene. But following this enzymatic cleavage the only ceramide obtained was lignocerylsphingosine; phosphoric acid and choline were also isolated, and the only free fatty acid found was palmitic acid. Since, in our experience, the amide-bound fatty acid in ceramides is not split by the enzyme preparation employed, the free palmitic acid was assumed to have been present as a fatty acid esterified with the second OH group of the sphingosine molecule.   To substantiate further the assumption that sphingomyelin occurs as a fatty acid ester, sphingomyelin was hydrolyzed with a purified pancreatic lipase.   Such an enzyme preparation could be expected to split off the fatty acid from the ester linkage but leave ceramide-bound fatty acid intact. Similarly, since the NH—CO bond is resistant to mild alkaline hydrolysis (7), saponification would liberate only the esterified fatty acid.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Enzymatic Hydrolysis with Liver Phosphatase*</div>

The enzymatic hydrolysis of large quantities of sphingomyelin presented several difficulties.   The enzyme preparation described by Rossi (8) proved unsatisfactory for our purposes, since it usually hydrolyzed not more than 40 per cent of the lipid.   Furthermore, the presence of bile salts previously believed to activate the enzyme was found to exert an inhibiting effect.   Although the sodium oleate which was used in preparing colloidal solutions of sphingomyelin proved to be an activator for the ferment, subsequent discovery of a fatty acid among the split-products precluded its use.

. For the preparation of the substrate, sphingomyelin (9) in 10 gm. quantities was shaken with about 50 cc. of water and sufficient ether to produce a uniform colloidal solution. This was added, drop by drop, with constant stirring, to about 500 cc. of water at 60°; as the ether was evaporated, the original volume was maintained by the addition of water. By this method colloidal solutions containing as much as 2 per cent sphingomyelin were prepared.

A very active enzyme preparation was obtained by washing 440 gm. of calf liver with tap water, grinding the tissue, and diluting it with an equal weight of water and with 44 gm. of an ethyl acetate-toluene mixture. The suspension was shaken mechanically for 3 hours and then placed in a water bath at 37° for 24 hours. From this material a clear filtrate of pH 4.5 to 4.7 was obtained which was brought to pH 7.0 by electrometric titration with N NaOH. The neutralized filtrate was further purified by 24 hour dialysis in cellophane sacs. The dialysate was centrifuged and the supernatant enzyme solution, when stored at refrigerator temperature, was stable for several weeks.

500 cc. of this purified liver phosphatase, 500 cc. of 0.1 N magnesium sulfate, and 500 cc. of glycocoll buffer (pH 8.9) were mixed and allowed to stand for 30 minutes in a water bath at 37°. To 1250 cc. of this mixture were added 19.9 gm. of sphingomyelin in solution, as described above. To prevent bacterial contamination, 1 to 2 cc. of toluene was added and the mixture was kept in a water bath at 37° for 3 weeks. The activity of the enzyme was manifested by the appearance, within 48 hours, of a flocculation in the upper layers. Phosphorus determinations with the Briggs method (10) served as a quantitative measure of the progress of the hydrolysis. During the course of the hydrolysis, the pH was corrected to 8.9 every 2 or 3 days by the addition of N NaOH. As a blank, 250 cc. of the enzyme-buffer-MgSO$_4$ solution were kept under identical conditions. At the end of 3 weeks, examination of the control enzyme solution failed to reveal the presence of any fatty acid. Choline and free phosphate were recovered, and these quantities were taken into consideration in calculation of the yield of the split-products.

The recovery of the products of hydrolysis was facilitated by acidifying with 50 cc. of 10 per cent trichloroacetic acid and precipitating with twice the volume of acetone (final pH 2.4). After storage in the refrigerator for 24 hours the white flocculent precipitate that formed was recovered by decanting the clear supernatant fluid and centrifuging the residue. The total residue was dried over P$_2$O$_5$ *in vacuo* to constant weight (13.6 gm.).

The filtrate was made slightly alkaline with N NaOH and freed of acetone by vacuum distillation. Then, after reacidification, the ether-soluble substances were removed, washed with water

until neutral, and dried for several days with anhydrous sodium sulfate. This ether-soluble fraction contained small amounts of fatty acids and was combined with the main fatty acid fraction obtained later. The ether-free filtrate was neutralized with N NaOH and concentrated *in vacuo* to a volume of 300 cc. When corrected for the values found in the enzyme solution blank, this filtrate, representing the water-soluble products of the hydrolysis of 19.9 gm. of sphingomyelin, contained (1) inorganic phosphate, 0.207 gm. of P $\backsimeq$ 0.655 gm. of phosphoric acid; (2) total phosphate, 0.391 gm. of P $\backsimeq$ 1.238 gm. of phosphoric acid; (3) free choline (determined as a reineckate), 5.204 gm. of reineckate $\backsimeq$ 1.434 gm. of choline; (4) total choline (determined as a reineckate following hydrolysis with concentrated HCl), 5.682 gm. of reineckate $\backsimeq$ 1.566 gm. of choline.

The residue (13.60 gm.) consisted of ceramide, fatty acid, and unsplit sphingomyelin. When ground with sea sand and extracted in a Soxhlet apparatus with the ether retained from the purification of the filtrate, the ceramide and fatty acid were completely removed, leaving the unsplit sphingomyelin. The latter, after purification, weighed 3.4 gm. (N 2.61, P 2.82 per cent).

The ceramide and fatty acid mixture, after evaporation of the ether, was extracted in a Soxhlet apparatus with acetone. On cooling, the ceramide was precipitated (7.442 gm., m.p. 88°). An additional 0.205 gm. of ceramide (m.p. 85°) was obtained by evaporating the acetone solution to dryness, redissolving in ether, and again cooling. This final precipitation removed all of the ceramide that had been kept in acetone solution by the fatty acid present. The analysis of this free fatty acid will be described later.

Ceramide Fraction—By hydrolysis of a portion of the ceramide (2.070 gm.) with 10 per cent methanol-sulfuric acid, fatty acid methyl ester and sphingosine sulfate were obtained. The fatty acid methyl ester was extracted with petroleum ether, washed until neutral, and dried *in vacuo*. After saponification, a fatty acid fraction was isolated (0.891 gm.), and following repeated recrystallizations from methanol and acetone melted at 76°. The molecular weight of the fatty acid was determined by titration (333) and was found to be lower than that calculated for lignoceric acid (368). Because of this deviation, some of the

ceramide (0.974 gm.) was hydrolyzed with methanolic sulfuric acid and the methyl esters were distilled. On saponification of the fractionated fatty acid methyl ester (0.520 gm.) about 0.095 gm. of palmitic acid (m.p. 61°) was recovered; the remainder of the distillate consisted of lignoceric acid (m.p. 82°).

Sphingosine sulfate was recovered from the methanolic sulfuric acid by evaporating the methanol and concentrating the solution on a water bath. On cooling, a coherent white mass separated, which was washed with ice water, dissolved in 0.5 N KOH, and extracted with ether. After drying, the free sphingosine weighed 0.880 gm., a recovery of 92.3 per cent. Sphingosine sulfate was prepared from the free base by neutralization with 0.2 N sulfuric acid in methanol solution.

Analysis—$(C_{18}H_{37}NO_2)_2 \cdot H_2SO_4 (696.7)$
Calculated. C 62.01, H 11.00, N 4.02
Found.   " 62.47, " 11.00, " 3.52

Fatty Acid Fraction—The impure free fatty acid that had been present with the ceramide was converted to the potassium soap with 0.5 N methanolic KOH. Following filtration, the soap was washed with cold ether and hydrolyzed with dilute sulfuric acid. The free fatty acid liberated was extracted with ether, and when dried weighed 2.56 gm. (m.p. 48°). By titration with alcoholic KOH the molecular weight of the impure product was found to be 286. It was possible to precipitate contaminating ceramides (0.079 gm., m.p. 84°) by redissolving in ether and cooling. After this purification the fatty acid melted at 54.5° and there was a corresponding decrease in the molecular weight (262). An additional 0.296 gm. of ceramide (m.p. 84°) was recovered from the mother liquor after removal of the potassium soaps. This was accomplished by evaporating the ether, diluting with water, and salting-out the cloudy emulsion with sodium chloride.

1.716 gm. of the pure fatty acid were dissolved in 50 times this weight of methanol containing 10 per cent sulfuric acid, and esterified on the water bath under a reflux for 5 hours. The reaction mixture was extracted with petroleum ether and, when dried, the methyl ester weighed 1.86 gm. Of this, 1.61 gm. were fractionally distilled. The fractions obtained were saponified with alcoholic KOH, acidified with dilute sulfuric acid, and extracted

with ether. The properties of the various fractions of the fatty acid are summarized in Table I. The iodine numbers were determined by the method of Rosenmund and Kuhnhenn (11).

The insignificant differences in the melting points and titration values of the various fractions suggest the presence of but one fatty acid. The low iodine number indicates the probability that the acid is saturated. These data closely approximate those of palmitic acid (mol. wt. 256.3; m.p. 62.3°).

Comment—Analysis of the products of enzymatic hydrolysis, corresponding to about 80 per cent of the original amount of sphingomyelin, yielded choline, phosphoric acid, ceramide (lignocerylsphingosine), and a fatty acid (palmitic acid). The residue

TABLE I

Distillation of Methyl Esters Derived from Fatty Acid Fraction

Fraction No.	Methyl ester			Fatty acid			
	Temperature of distillation	Pressure	Weight	Weight	I No.	Mol. wt.	M.p.
	°C.	mm. Hg	gm.	gm.			°C.
1	90– 94	0.01	0.033	0.081	6	251	58–59
2	93– 96	0.01	0.069				
3	96–102	0.01	0.207	0.201	8	261	59–60
4	102–108	0.01	0.155	0.639	4	262	59–60
5	104–110	0.01	0.515				
6	110–114	0.02	0.292	0.234	3	263	60–61
7	113–111	0.02	0.186	0.179	6	269	60–61

of unhydrolyzed sphingomyelin contained N 2.61, P 2.82 per cent. These values more closely approximate those of lignocerylsphingomyelin palmitic ester (mol. wt. 1071.3; calculated, N 2.61, P 2.89 per cent) than those of lignocerylsphingomyelin (mol. wt. 832.84; calculated, N 3.36, P 3.73).

Hydrolysis of 0.974 gm. of the ceramide fraction demonstrated that it consisted mainly of lignocerylsphingosine. The recovery of 0.095 gm. of palmitic acid may be explained in one of two ways. The acid may have been bound to the sphingosine in the form of an amide (palmitylsphingosine), in which case the ceramide fraction would have consisted of two different sphingosine amides. Comparison with synthetic sphingosine amides

(7) makes this possibility unlikely, although the findings do not exclude the existence of a palmitylsphingosine. The other possibility is that the palmitic acid was present in the form of a lignocerylsphingosine fatty acid ester, and that the ceramide fraction was a mixture of this ester and lignocerylsphingosine. The following experiments lend support to this view.

Enzymatic Hydrolysis with Pancreatic Lipase

Glycerol extracts of powdered pancreas contain a highly active lipase. However, such preparations also exhibit some phosphatase activity and the glycerol interferes with the isolation of the products of hydrolysis. To eliminate these undesirable effects 50 cc. of a 1:16 glycerol extract of dried pancreas (12) were diluted with an equal volume of ice water and treated with

TABLE II

Lipase Activity before and after Acetone Precipitation

Hydrolysis for 1 hour; pH 8.9.

Enzyme preparation		Enzyme	Titration value	Hydrolysis	Lipase
		cc.	cc. 0.05 N NaOH	per cent	units per cc.
Glycerol extract		0.08	2.34	9.7	4.17
Acetone pptn.		0 50	4.60	21.6	1.49

200 cc. of acetone. The precipitate containing the lipase was permitted to settle out at refrigerator temperature and then centrifuged. The centrifugate was washed three times with 50 cc. portions of 66 per cent acetone, after which it was redissolved in 50 cc. of physiological saline and freed from remaining traces of acetone by bubbling air through the solution. The final preparation was stored in the refrigerator; a small amount of toluene (0.5 cc.) was added to prevent bacterial contamination.

The lipase activity of the preparation before and after acetone precipitation was determined by the Vogel and Laeverenz modification (13) of the Willstätter, Waldschmidt-Leitz, and Memmen method (12). The results are shown in Table II.

50 cc. of the enzyme preparation were incubated at 37° for 1 hour with 1.0 gm. of calcium chloride and 0.5 gm. of albumin

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washed with ether and converted to the methyl ester by esterification with 10 per cent sulfuric acid in methanol. The methyl ester was extracted with petroleum ether, dried with anhydrous sodium sulfate, and fractionally distilled *in vacuo*. The fatty acids were recovered by saponification with alcoholic KOH, acidified with dilute sulfuric acid, and extracted with ether. The values obtained are shown in Table III and are very close to those expected for palmitic acid.

The control solution of enzyme mixture, without substrate, was treated in the same manner, and the total fatty acid fraction was equivalent to 4.96 cc. of 0.1 N NaOH. If it is assumed that this titration value is due to the presence of a fatty acid and that the latter consists of a 1:1 mixture of palmitic and stearic acids

TABLE III

Distillation of Methyl Esters Derived from Fatty Acid Fraction

Fraction No.	Methyl ester		Fatty acid			
	Temperature of distillation (pressure 0.01 mm. Hg)	Weight	Weight	I No.	Mol. wt.	M.p.
	°C.	gm.	gm.			°C.
1	90–100	0.082	0.063	10.4	254	56–58
2	97–100	0.215	0.197	11.5	252	57–58
3	98–97	0.026	0.023	4	251	59

(average molecular weight 270), the filtrate contains 0.134 gm. of fatty acid.

Saponification of Sphingomyelin with Alkali

The method employed is based upon the procedure described by Henriques (14) for the hydrolysis of tripalmitin and tristearin. 1.0 gm. of sphingomyelin was dissolved in 5 cc. of methanol, 45 cc. of petroleum ether, and 20 cc. of 0.5 N methanolic KOH. After standing at room temperature for 48 hours, the methyl ester was extracted with several portions of petroleum ether. When washed, dried, and evaporated to dryness, the methyl ester weighed 0.139 gm. The methanolic portion containing the saponified sphingomyelin was evaporated almost to dryness. 50 cc. of ether were added to the residue and the phosphatide precipi-

tated with an excess of acetone (250 cc.). The white flocculent precipitate was washed with acetone and after drying weighed 0.673 gm.

Analysis—Lignocerylsphingomyelin; $C_{47}H_{97}N_2PO_7$ (832.8)
Calculated, N. 3.36, P 3.73; found, N 3.32, P 3.97

The acetone-insoluble Reinecke salt obtained from this lignocerylsphingomyelin contained N 10.25, P 2.72 per cent. These values closely approximate those for a Reinecke complex consisting of 1 molecule of lignocerylsphingomyelin and 1 molecule of Reinecke acid (N 9.73, P 2.69).

<div align="center">TABLE IV</div>

Saponification Values of Different Sphingomyelins and of Other Fatty Acid Ester Compounds

Substance	Weight	Time for saponification	0.5 N alcoholic KOH	
			Calculated	Found
	gm.	*hrs.*	*cc.*	*cc.*
Tripalmitin......................	0.200	4	1.49	1.36
Tristearin.......................	0.200	4	1.34	1.24
O-Dipalmityllignocerylsphingosine...........................	0.200	1	0.71	0.73
O-Distearyllignocerylsphingosine .	0.200	1	0.68	0.67
Sphingomyelin (liver)*............	0.400	4	0.75	0.60
" (spleen)*..........	0.300	4	0.56	0.61

* Calculated for lignocerylsphingomyelin palmitic acid ester.

According to Henriques, this method of saponification yields a soap in addition to the methyl ester. Therefore, the supernatant fluid obtained from the acetone precipitation of the saponified sphingomyelin was brought to dryness and added to the methyl ester fraction. The total quantity was saponified with 15 cc. of 0.5 N methanolic KOH on a water bath for 3 hours, diluted with water, and acidified with 10 per cent sulfuric acid. The fatty acids were extracted by shaking with ether, washed with water, and dried; yield 0.157 gm. After repeated ether purifications, the titration of the fatty acid with 0.5 N methanolic KOH corresponded to a molecular weight of 250.2.

Samples of sphingomyelin from liver and spleen were saponified

and the fatty acid yield was titrated in a water-free medium. Although the saponification was carried out for 4 hours on the water bath, the procedure should be limited to 1 hour, since it is possible that boiling for a longer time may result in a slight decomposition of the sphingomyelin molecule. The values obtained are presented in Table IV. Included for comparison are the saponification values of tripalmitin, tristearin, O-dipalmityllignocerylsphingosine (7), and O-distearyllignocerylsphingosine (7).

Saponification of Ketene-Acetylated Sphingomyelin

A method for determining the ratio of esterified to free sphingomyelin was suggested by experiments in which an ether-soluble O-diacetyllignocerylsphingosine was formed by the ketene acetylation of the ceramide (7). Consequently, 0.108 gm. of sphingomyelin (spleen) was acetylated with ketene and the products were saponified for 72 hours. Saponification of 0.108 gm. of the unacetylated lipid blank required 0.69 cc. of 0.1 N KOH, whereas the acetylated sphingomyelin mixture required 1.09 cc. of 0.1 N KOH. Assuming one unesterified lignocerylsphingomyelin (833 mol. wt.) to combine with one acetyl group (42 mol. wt.), complete saponification would require 1.23 cc. of 0.1 N KOH for titration Therefore, 67.5 per cent of the sphingomyelin prepared from spleen is esterified.

DISCUSSION

In our early experiments, the presence of a fatty acid among the split-products of the enzymatic hydrolyses was entirely unexpected. Since neither of the enzyme preparations employed split the ceramide lignocerylsphingosine, it is unlikely that palmitylsphingosine or stearylsphingosine would be hydrolyzed by these ferments. If the recovered palmitic acid is not present as a ceramide-bound fatty acid, it is most probable that the sphingomyelin is esterified.

The facts presented lead to the conclusion that the sphingomyelin examined consisted of a mixture of free and esterified phosphatide. However, there is the possibility that the lipid exists solely in the esterified form in nature, and that partial autolysis of the ester takes place in the tissue during the process of isolation. Several authors have found (15) the nitrogen and

phosphorus values to be lower in brain sphingomyelin than in sphingomyelin isolated from spleen and liver. This suggests the possibility that the esterified form of the lipid predominates in brain. The negligible lipase activity of brain tissue compared with liver (16) also points in this direction. Studies on brain sphingomyelin are now in progress.

SUMMARY

1. Sphingomyelin from spleen was hydrolyzed with a liver enzyme, a purified pancreatic lipase, and by the Henriques method of alkaline saponification.

2. With the liver enzyme 80 per cent of the sphingomyelin was split, yielding choline, phosphoric acid, palmitic acid, and lignocerylsphingosine. Cholinephosphoric acid was not isolated as such, but there was evidence of its presence among the split-products.

3. When pancreatic lipase was used, the end-products isolated were palmitic acid, lignocerylsphingomyelin, and a small amount (13 per cent) of unsplit lignocerylsphingomyelin ester.

4. Saponification of sphingomyelin resulted in palmitic acid and a phosphatide identified as unesterified lignocerylsphingomyelin.

5. The presence of a free fatty acid among the split-products of enzymatic hydrolysis can be explained only by assuming the existence of a sphingomyelin in which the second OH group of the sphingosine is esterified.

6. An acetylation method is presented for determining the amount of unesterified lipid present in sphingomyelin preparations.

7. Sphingomyelin probably exists as a mixture of lignocerylsphingomyelin and lignocerylsphingomyelin fatty acid ester.

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STUDIES ON ANIMAL LIPIDS

XVII. THE SYNTHESIS OF LIGNOCERYLSPHINGOSINE FATTY ACID ESTERS (SPHINGOSINE FATS) AND SPHINGOSINE AMIDES (CERAMIDES)*

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The synthesis and study of the hitherto unknown lignocerylsphingosine fatty acid esters were prompted by the desire to compare their physical and chemical properties with those of sphingomyelin fatty acid ester. The successful preparation of these derivatives would provide a previously undescribed group of chemical substances, sphingosine fats, of possible biological significance. By the same methods, attempts were made to synthesize sphingosine fats with the same fatty acid in both the ester and acid amide (CO—NH) linkages. Since the CO—NH bond is resistant to mild alkaline saponification, the hydrolysis of such compounds should liberate the ester fatty acid, leaving the sphingosine amide fatty acid intact. Although thus far only one sphingosine amide (lignocerylsphingosine) has been found as such in nature (1, 2), other ceramides are considered to be a part of the cerebroside and sphingomyelin molecules (3). Knowledge of the physical and chemical properties of the synthetic ceramides should be of aid in studying the sphingosine amides in nature.

EXPERIMENTAL

O-Diacetyl-, O-dipalmityl-, O-distearyl-, and O-dibenzoyllignocerylsphingosine were prepared. The acetylation was carried out with ketene ($H_2C{=}C{=}O$), the gas being prepared according to the method of Herriott (4). 245.3 mg. of ketene (an acetic acid value of 350.4 mg.) were obtained by use of 110 volts D.C.

and 5 amperes for 90 minutes.   The acetyl determinations were carried out by the method of Kuhn and Roth (5).

O-Diacetyllignocerylsphingosine—1.0 gm. of lignocerylsphingo-sine was dissolved in a special reaction flask containing a mixture of 20 cc. of dried chloroform and 1 cc. of 0.2 N methanolic KOH as a catalyst.   The flask was connected to the ketene apparatus and the gas bubbled through for 2 hours.   The chloroform solu-tion was evaporated to dryness and the residue dissolved in 100 cc. of ether.   This was washed with water until neutral, dried with anhydrous sodium sulfate, and the ether evaporated *in vacuo*.   0.90 gm. was obtained which was readily soluble in alcohol, chloroform, and ether, but less soluble in acetone.   The melting point was 70–71°.

Analysis—$C_{46}H_{87}NO_5$(733.7).   Calculated.   C 75.24, H 11.96, N 1.91
　　　　　　　　　　　　　　　Found.　　" 75.24, " 12.20, " 2.04

Acetyl Determination—Alkaline saponification, 2 hours
　　Calculated.   32.3 mg. substance = 2.64 cc. 0.3 N NaOH
　　Found.　　　32.3 "　　　　" 　　= 2.55 " 0.3 " 　"

Molecular Weight Determination—
　　1.070 mg.; 7.670 mg. camphor; depression 8.7°, 7.7°; 642, 720
　　0.795 " 　4.830 "　　　"　　　　　"　　 9.7°, 9.2°; 680, 715

O-Dipalmityllignocerylsphingosine—The method employed was based on the esterification procedure of Einhorn and Holland (6) with quinoline.   A mixture of palmityl chloride (3 cc.) and sodium-dried ether (10 cc.) was dropped slowly into a reaction flask containing 0.910 gm. of lignocerylsphingosine dissolved in 5 cc. of quinoline (dried with BaO).   The water-free reaction mixture was then warmed on a water bath, under a reflux, for 4 hours.   After cooling, 100 cc. of ether were added, and the quinoline was removed by shaking with 2 N HCl.   The traces of HCl were removed by washing with water; the neutral reaction mixture was dried with anhydrous sodium sulfate and treated with a 10-fold volume of methanol.   A straw-colored oil which sepa-rated was dissolved in ether and again treated with methanol. The oil which reformed was separated from the supernatant fluid, redissolved in 25 cc. of ether, and added drop by drop with constant shaking to 300 cc. of cooled methanol.   The white flocculent precipitate was separated by centrifuging, washed with

cooled methanol, and when dried weighed 1.164 gm. (74 per cent of the theoretical yield). The substance was readily soluble in acetone, ether, and chloroform, but only slightly soluble in hot alcohol. The fat crystallized from an ether-methanol solution in bundles of needles which melted at 39–40°.

Analysis—$C_{\text{t}}H_{\text{t6}}NO_4$(1126.14). Calculated. C 78.86, H 12.80, N 1.27
                                        Found.   " 79.07, " 12.43, " 1.42
                                                    " 78.86, " 12.36

Alkaline Saponification—1 hour; 0.200 gm.
   Calculated (for two fatty acid radicals) = 0.71 cc. 0.5 N KOH
   Direct titration (blank test)   ..... = 0.02 " 0.5 " "
   Main test.. .... = 0.75 " 0.5 " "

Molecular Weight Determination—
   0.820 mg.; 5.370 mg. camphor; depression 4.7°, 5.2°; 1300, 1170
   0.825 " 2.935 "   "   "   9.7°, 9.2°; 1160, 1220

O-Distearyllignocerylsphingosine—The synthesis and purification of this derivative were carried out in the same manner as described above. 1.0 gm. of lignocerylsphingosine and 3 cc. of stearyl chloride were used. The product weighed 1.20 gm. and melted at 45–47°. It was easily soluble in acetone, ether, and chloroform, but slightly soluble in hot alcohol.

Analysis—$C_{\text{74}}H_{\text{145}}NO_4$(1182.2). Calculated. C 79.17, H 12.87, N 1.18
                                     Found.   " 79.04, " 12.66, " 1.42

Alkaline Saponification—1 hour; 0.200 gm.
   Calculated (for two fatty acid radicals) = 0.68 cc. 0.5 N KOH
   Direct titration (blank test)... .. .. = 0.02 " 0.5 " "
   Main test..... .   . . . = 0.69 " 0.5 " "

Molecular Weight Determination—
   0.790 mg.; 5.330 mg. camphor; depression 5.7°, 4.9°; 1040, 1210
   0.700 " 6.250 "   "   "   4.0°, 3.7°; 1120, 1210

O-Dibenzoyllignocerylsphingosine—The procedure for the preparation of this derivative followed the method outlined for the synthesis of O-dipalmityllignocerylsphingosine up to the step where the neutral reaction mixture was dried. The latter was then evaporated almost to dryness in a centrifuge tube and treated with 50 cc. of cooled methanol. The precipitate that formed on cooling was separated by centrifuging, redissolved in hot methanol, and placed in the refrigerator. This procedure

was repeated until all of the excess benzoic acid was removed as evidenced by a neutral reaction of the alcoholic solution. The product weighed 1.045 gm. and melted at 57–58°. It was highly soluble in ether, acetone, and chloroform and soluble in hot alcohol. The fat crystallized from ether-methanol solution in clusters resembling bur-like filamentous tufts.

Analysis—$C_{56}H_{91}NO_5$(857.7). Calculated.   C 78.34, H 10.70, N 1.63
                             Found.   " 78.58, " 10.48, " 1.82

Molecular Weight Determination—

   0.950 mg.; 7.005 mg. camphor; depression 6.5°, 6.3°; 840, 861
   0.755 "   6.505 "   "   "   5.5°, 5.6°; 844, 829

Tripalmitylsphingosine—By the same procedure, with 0.50 gm. of sphingosine and 3 cc. of palmityl chloride, 1.175 gm. of tripalmitylsphingosine were obtained. The product was very soluble in acetone, ether, and chloroform, but only slightly soluble in hot alcohol. The crystalline compound melted at 67–69°.

Analysis—$C_{68}H_{135}NO_5$(1014). Calculated.   C 78.00, H 12.60, N 1.40
                             Found.   " 77.88, " 12.71, " 1.22

Molecular Weight Determination—

   1.040 mg.; 5.005 mg. camphor; depression 8.2°, 8.2°; 1014, 1014
   0.885 "   6.245 "   "   "   5.5°, 5.2°; 1030, 1090

Tristearylsphingosine—From 0.450 gm. of sphingosine and 3 cc. of stearyl chloride, 1.345 gm. of tristearylsphingosine were prepared. The product melted at 72–74° and was very soluble in acetone, ether, and chloroform, but slightly soluble in hot alcohol.

Analysis—$C_{72}H_{139}NO_5$(1098.2). Calculated.   C 78.68, H 12.76, N 1.27
                             Found.   " 78.19, " 12.81, " 1.44

Molecular Weight Determination—

   0.750 mg.; 4.800 mg. camphor; depression 5.8°, 5.6°; 1080, 1116
   0.775 "   4.250 "   "   "   6.8°, 7.0°; 1073, 1042

Tribenzoylsphingosine—From 0.600 gm. of sphingosine and 2 cc. of benzoyl chloride, 0.935 gm. of tribenzoylsphingosine was prepared. The product crystallized from methanol in long colorless needles and melted at 118–120°. It was very soluble in acetone, ether, and chloroform and soluble in hot alcohol.

Analysis—$C_{39}H_{49}NO_5$(611.39). Calculated.   C 76.54, H 8.08, N 2.29
                             Found.   " 76.10, " 8.25, " 2.15

Molecular Weight Determination—

    0.620 mg.; 4.465 mg. camphor; depression 8.7°, 8.7°; 638, 638
    0.695 "   4.700 "   "   "   9.3°, 9.4°; 636, 629

By saponification of the sphingosine fats with methanolic KOH and extraction of the dried material with sodium-dried ether in a Soxhlet apparatus, the ceramides palmitylsphingosine and stearylsphingosine were prepared. The isolation of palmitylsphingosine, as described below, will serve as an illustration of the method employed.

*Palmitylsphingosine—*0.915 gm. of tripalmitylsphingosine was treated with 20 cc. of 0.5 N methanolic KOH in the presence of enough ether to dissolve the alcohol-insoluble sphingosine fat, and permitted to stand at room temperature for 48 hours. The reaction mixture was dried with a fan at room temperature, ground with sea sand, and extracted with sodium-dried ether in a Soxhlet apparatus. The ether solution, containing only ceramide, was evaporated to dryness and the residue dissolved in methanol. The white, flocculent, crystalline precipitate which settled out on standing in the cold was filtered, washed with cooled methanol, and dried. After repeated crystallizations from methanol the product formed clusters of large round disks with radiations and melted at 86–87°. 0.366 gm. was recovered, which was soluble in ether and chloroform in the cold, soluble in hot methanol and acetone.

Analysis—$C_{34}H_{67}NO_3$(537.57). Calculated. C 75.90, H 12.56, N 2.61
                                  Found.   " 76.26, " 12.75, " 2.54

Molecular Weight Determination—

    0.730 mg.; 4.000 mg. camphor; depression 14.7°, 14.5°; 496, 503
    0.625 "   4.150 "   "   "   12.0°, 11.7°; 502, 515

*Stearylsphingosine—*0.987 gm. of tristearylsphingosine hydrolyzed in 20 cc. of methanolic KOH yielded 0.325 gm. of stearylsphingosine. The derivative, when crystallized from methanol, formed clusters of small hooks, melting at 88–89°. The ceramide was soluble in ether and chloroform in the cold, and in hot acetone and methanol.

Analysis—$C_{36}H_{71}NO_3$(565.6). Calculated. C 76.37, H 12.60, N 2.47
                                    Found.   " 76.11, " 12.63, " 2.27

Molecular Weight Determination—

    0.580 mg.; 4.050 mg. camphor; depression 10.5°, 10.7°; 547, 535
    0.675 "   3.950 "   "   "   12.6°, 12.4°; 542, 551

DISCUSSION

The crystalline fatty acid esters of ceramides constitute a hitherto undescribed group of chemical substances for which the name sphingosine fats is suggested.

The chemical and physical properties of the lignocerylsphingosine fatty acid esters resemble very closely those of the triglycerides. They are very soluble in ether, acetone, and chloroform, and are almost insoluble in hot alcohol. These properties differ markedly from those of their components (lignocerylsphingosine and fatty acid) and facilitated the purification of the compounds.

The readiness with which the sphingosine fats are saponified provides a method for preparing synthetic ceramides.

The synthesis of these compounds demonstrates that the esterification of both hydroxyl groups of the sphingosine in the ceramide is possible. That the sphingosine fats may have some biological significance is suggested by their relationship to sphingomyelin and cerebroside; *i.e.*, (*a*) lignocerylsphingosine + cholinephosphoric acid = sphingomyelin; (*b*) lignocerylsphingosine + galactose = cerebroside; (*c*) lignocerylsphingosine + fatty acid = sphingosine fat.

Analyses reported in this and in Paper XVI were performed by the Dr. C. Tiedecke Laboratory for Microchemistry, New York.

SUMMARY

1. The preparation and properties of lignocerylsphingosine fatty acid esters (sphingosine fats) and sphingosine amides (ceramides) are described.

2. The synthesis of these compounds demonstrates that the esterification of both hydroxyl groups of the sphingosine in the ceramide is possible.

3. The readiness with which the sphingosine fats are saponified provides a method for preparing synthetic ceramides.

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AN IMPROVED METHOD FOR THE DETERMINATION OF NON-HEMIN IRON

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From the chemical point of view, biological iron compounds can only be classified as follows: (1) hemin (or heme) compounds such as hemoglobin, cytochrome, catalase, peroxidase, or respiratory enzyme, whose formula is more or less well established in so far as the hemin group is concerned; (2) all others, *i.e.* non-hemin iron compounds, whose chemical nature, in spite of some hopeful attempts (Smythe and Schmidt (1), McFarlane (2), Tompsett (3), and particularly Fischer and Hultzsch (4)), must be considered as unknown to date. Nevertheless, quantitative determinations of the latter group are very often performed by workers in physiology and biochemistry, and considerable effort has been spent in establishing satisfactory methods. Table I summarizes the methods developed for the direct determination of non-hemin iron in the presence of hemin compounds. Most of these have been criticized and have from time to time undergone improvements, particularly the so called "bipyridine method."

Generally speaking, an effective method of extraction must suit two requirements: the extraction of the non-hemin iron must be exhaustive, and no hemin iron must be extracted. The best way to test this is to compare the method in question with an indirect one, in which the non-hemin iron is calculated from the difference between total iron and hemin iron. The following modification of one of the methods (6) has been carefully tested by comparison with direct and indirect methods and enables the rapid and quantitative determination of non-hemin iron in tissues and other biological materials in the normally equipped laboratory.

EXPERIMENTAL

Tompsett (6) extracted the non-hemin iron by treating the tissue for 30 minutes with sodium pyrophosphate and trichloroacetic acid.[1]   As has been found by Tingey (11) and the authors (12), complete extraction requires far more time, from 1 to 3 days. We have now found that the same effect can be reached simply by heating for 7 minutes at 100°.   Table II illustrates the efficiency of this procedure as compared with the former ones.   Table III shows that recovery of added Fe^{+++} is satisfactory.   Table IV

TABLE I

Methods Used for Direct Determination of Non-Hemin Iron in Tissues

Author	Non-hemin iron extracted by	Finally determined with
Starkenstein and Weden (5), 1928	5 N HCl	Thiocyanate or iodometric method
Tompsett (3, 6), 1934–35	Thioglycolic acid, or $Na_2S_2O_4$, or $Na_4P_2O_7$	Thioglycolic acid
Hill (7), 1930	Reducing agents + bipyridine	α,α'-Bipyridine
Shackleton and McCance (8), 1936	$Na_2S_2O_4$ + bipyridine or $Na_2S_2O_4$ alone	"
Kohler, Elvehjem, and Hart (final modification) (9), 1936	Hydroquinone + bipyridine	"
Borgen and Elvehjem (10), 1937	Homogenization and heat	"

gives the results of the test determinations.   After being minced in the usual way, each sample listed in Table IV was analyzed by the following methods.

Hot Pyrophosphate Method—1 gm. of the tissue pulp is ground in a mortar with powdered glass, a few cc. of saturated sodium pyrophosphate, and 10 per cent trichloroacetic acid (purified by distillation *in vacuo*), and then quantitatively transferred to a wide centrifuge tube.   The usual amounts were 5 cc. of $Na_4P_2O_7$ and 10 cc. of CCl_3COOH.   The tube is then heated in a boiling

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[1] The chemical mechanism of the interesting reaction has been studied by us in detail, the results of which will be presented in another paper.

water bath for exactly 7 minutes, immediately centrifuged, and the residue washed twice with 4 cc. of an equal mixture of the two reagents. The united extracts form a clear and practically colorless liquid which is ready for determination. A faint yellowish shade (flavins?), or a slight opalescence, which sometimes occurs, disappears during the following treatment with ammonia and reducing agents.

TABLE II

Influence of Time and Temperature on Extraction of Non-Hemin Iron with Pyrophosphate

	Liver 105			Liver 116	
	Fe extracted				Fe extracted	
Time treated	Sample A, 25°	Sample B, 25°	Time treated	Sample A, 25°	Sample B, 100°
	γ	γ		γ	γ
30 min.	167	138	7 min.		438
1 day		668	30 "	198	
2 days		730	2 days	368	
4 "	717	742	3 "	417	
4½ "	730	744	4 "	434	
			Finally heated for 5 min., 100°	442	453

TABLE III

Recovery of Fe^{+++} Added to Tissue (Hot Pyrophosphate Method)

Tissue	Initial content	Fe added	Non-hemin Fe found	Non-hemin Fe calculated
	γ	γ	γ	γ
Liver.....	229	121	344	350
Kidney..	36	110	144	146

Iron determinations were carried out in aliquot parts of the filtrate with thioglycolic acid or *o*-phenanthroline (now used by most workers instead of the more expensive α,α'-bipyridine). For routine work we have nearly always used thioglycolic acid, but the values given in Table IV are mean values of two determinations with thioglycolic acid and two with *o*-phenanthroline, which never showed a difference of more than 4 per cent.

Details of the respective techniques have so often been described that we wish to confine ourselves to the following remarks. The thioglycolic acid technique was carried out according to Tompsett (3); before the reading was taken, the red solution was shaken to prevent fading of the color. *o*-Phenanthroline was used the same way as bipyridine according to Lintzel (13); the pH was adjusted with *p*-nitrophenol as indicator, and sodium hydrosulfite ($Na_2S_2O_4$, not $Na_2S_2O_3$) was used as the reducing agent. Full color developed after 15 minutes standing. Some values were also controlled with the thiocyanate method carried

TABLE IV

Non-Hemin Iron Determined by Different Methods

The results are expressed in mg. per kilo of dry tissue.

Sample	Pyrophosphate at 100°	Pyrophosphate at 25°	HCl method	Total iron	Hemin iron		Non-hemin iron calculated
					Colorimetric	Direct	
	(1)	(2)	(3)	(4)	(5)	(6)	(4) − (6)
1. Kidney, man..........	220	227	230	434	344	206	228
2. Liver, "	981	1012	1050	1100	130	125	975
3. Kidney, "	189	196	135		798	772	
4. Liver, rat............	674	701	600	960	285	270	690
5. Kidney, man..........	118	128	128	295	192	176	119
6. Testis, "	160	145	150	178	18	18	160
7. Liver, calf............	357	360	373	637	285	290	347
8. " man*.....	535			590	98	60	530

* Expressed in mg. per kilo of fresh tissue.

out according to Kennedy (14). Since pyrophosphates interfere with the reaction, they must first be converted into orthophosphate, which was done by wet ashing with sulfuric and perchloric acids.

To control the completeness of the extraction, the residue was heated once more with pyrophosphate and trichloroacetic acid. Occasionally another small amount of Fe appeared, which never exceeded 5 per cent of the total non-hemin Fe. In the figures reported in Table IV, this amount is included; for practical purposes, however, it can be neglected. Further heating with pyrophosphate remained without effect.

All colorimetric work was performed with an extinction photom-

eter (Leitz), with Filter 495 mμ. Its chief advantage over instruments of the Duboscq type consists in the possibility of analyzing even turbid extracts.

Cold Pyrophosphate Method—The procedure was carried out as for the hot pyrophosphate method except that the centrifuge tube was left for 3 days at room temperature instead of being heated. The determinations and control extraction are performed as under the method above.

Hydrochloric Acid Method (Starkenstein and Weden (5))—1 gm. of the pulp is treated as in the first method, but 10 cc. of 5 N HCl are used instead of pyrophosphate and trichloroacetic acid. After 7 minutes heating, 5 cc. of 20 per cent CCl_3COOH are added, and the mixture is centrifuged and washed twice with 4 cc. of 10 per cent CCl_3COOH. The united extracts are more or less brown in color, a fact which complicates estimation with Duboscq instruments. Analysis and control extraction are carried out as in the methods above.

Total Iron—Fresh or dried samples of the tissue pulp were ashed with H_2SO_4 and $HClO_4$ and the iron content determined with thioglycolic acid, *o*-phenanthroline, and sometimes thiocyanate. Again, the single values agreed very closely; in Table IV the mean values are given.

Hemin Iron (Yabusoe (15))—1 gm. of tissue pulp is ground with powdered glass, 1 cc. of N HCl, and a few cc. of ice-cold absolute methanol, transferred to a centrifuge tube, and centrifuged and washed with methanol until the washings are colorless. The united filtrates are shaken with 1 gm. of powdered magnesium sulfate (iron-free) and after 30 minutes centrifuged. The filtrate is clear, light or dark brown,[2] and ready for analysis. This was carried out by comparing the color photometrically against a hemin standard solution (Filter 620 mμ), and, for control, by ashing aliquot parts of the extract and determining the iron con-

[2] An interesting observation may be noted. Very occasionally—not in the cases listed above—the hemin extracts had a green or olive color. Such abnormal extracts contained a large amount of non-hemin iron, which increased on standing, accompanied by fading of the color, and which could be precipitated along with the proteins by addition of $MgSO_4$. We did not study the phenomenon further but think that it is closely related to the observations on bilirubinoid substances made by Barkan (16) and Lemberg (17) and their coworkers.

tent of the ash solution. As previously reported (12) and as can be seen from Table IV, the colorimetric procedure sometimes gave higher values than the direct, probably owing to the presence of other iron-free pigments. The values for non-hemin iron were calculated on the basis of the figures obtained by the direct method.

It is evident from Table IV that the two pyrophosphate methods check very closely with the indirect one, whereas the results with the HCl method are more inconsistent.

If blood is treated with boiling pyrophosphate and trichloroacetic acid, increasing amounts of iron are extracted, ranging from about 2 mg. per cent after 7 minutes to 10 mg. per cent and

TABLE V

Effect of $Na_4P_2O_7$ and Heat on Recovery of Fe^{++} and Fe^{+++} in Various Concentrations of Acid

The samples were heated 10 minutes and then centrifuged.

CCl₃COOH	Saturated Na₄P₂O₇ added	Fe present	Fe recovered	
			Thioglycolic acid	o-Phenanthroline
per cent	*cc.*	γ	γ	γ
0	2	50 (Fe^{+++})	49.5	50.6
2.5	2	50 "	49.7	49.2
10	1	50 "	50.9	50.1
15	2	50 (Fe^{++})	51.2	50.0

more after 3 hours. Pure hemin is practically not attacked. The method is thus not applicable for the determination of the "easily split off" blood iron, in contrast to the cold pyrophosphate method which gives definite final values after 24 hours in the order of 1.6 mg. per cent for human blood (12). If blood is in contact with tissue, however, both procedures give nearly identical results, even in those rare cases in which, owing to abnormally high amounts of blood, far more hemin iron is present than non-hemin iron (Table IV, Sample 3). In contrast to blood alone, the hemin molecule seems not to be attacked here and the extraction reaches a final fixed value, enabling a sharp separation of hemin and non-hemin iron.

Some final remarks must be made concerning the statement of

Borgen and Elvehjem (10) that heating of small amounts of iron with $Na_4P_2O_7$ leads to "the formation of acid-insoluble iron pyrophosphate which remains in the residue upon centrifuging." As is well known, a very stable, complex iron-pyrophosphate anion of the type $[Fe_2(P_2O_7)_3]''''''$ which is soluble in water and acids is readily formed under the given conditions, and it is just this reaction the pyrophosphate method is based on. We have therefore carefully repeated the experiments described in Table IV of the paper of Borgen and Elvehjem (10). After solutions of Fe^{++} and Fe^{+++} were heated in a boiling water bath with saturated $Na_4P_2O_7$ at different concentrations of CCl_3COOH, the mixture was centrifuged and the iron content of the filtrates determined with thioglycolic acid or o-phenanthroline, as described above. The results, listed in Table V, show that in every case complete recovery was possible, contrary to the findings of the Wisconsin authors.

SUMMARY

1. When tissue is heated for 7 minutes at 100° with sodium pyrophosphate and trichloroacetic acid, all of the non-hemin iron is extracted.

2. The method has been tested in a number of cases by comparison with several others. It gives values for non-hemin iron which check very closely with those obtained by indirect determination of this fraction.

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CAROTENOIDS OF HUNGARIAN WHEAT FLOUR

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Whether or not wheat flour contains provitamin A in amounts which could be of any importance for general nutrition is a question of considerable interest. Since Wesener and Teller (1) as well as Monier-Williams (2) considered carotene, $C_{40}H_{56}$, to be the chief fat-soluble pigment of flour, this claim has been more or less supported by numerous authors (3). It was, however, correctly pointed out in the short reviews given by Widmark and Neymark (4) and by Jörgensen (5) that the reported carotene content of flour decreased with increased progress in this field. While Ferrari and Bailey (6) considered "carotin" the chief component of the coloring matter, it is rightly stated in an important paper by Markley and Bailey (7) that only a fraction of the wheat carotenoids can consist of carotene; e.g., one-third to one-seventh of the total pigment.

These figures are, however, still much too high. Malmberg and von Euler (8) were unable to detect any carotene at all in 100 gm. of wheat, and they found xanthophyll, $C_{40}H_{56}O_2$, to be the chief polyene, in accordance with the data of Bowden and Moore (9) who proved the presence of xanthophyll in wheat germ oil.

According to our new chromatographic experiments, only a small part of the carefully saponified pigment shows an epiphasic behavior, and even this fraction can easily be separated from added β-carotene in the Tswett column. Consequently we claim that the analyzed, unbleached flours are practically or absolutely free of carotene, the carotene content being less than 0.01 mg. per kilo in any case. Other samples are under investigation, but even our whole wheat flour does not contain much more carotene or xanthophyll than fine grade samples.

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As far as we know, no carotenoid has yet been isolated in crystallized form from flour, obviously because of the small amounts of the pigment present and of the very unfavorable proportion between coloring matter and colorless substances in the extracts. Earlier investigations carried out with animal fats in this laboratory showed, however, that in similar cases a sequence of several chromatographic operations may gradually improve the degree of purity, and finally allow the separation of the pigment. By applying this principle, we isolated 15 mg. of well crystallized, pure xanthophyll (lutein) from 60 kilos of flour.

EXPERIMENTAL

Chromatographic Analysis of Flour Carotenoids

Carotene—The starting material was unbleached, fine grade wheat flour from southern Hungary ("zero flour") which was harvested in July and milled in October, a few days before the experiments were begun. In the course of 4 to 5 hours 1 kilo was percolated with peroxide-free ether, whereupon the extract (1.5 liters) was kept for a night over 0.1 liter of concentrated methanolic KOH. The dark alkaline layer was then drained off, gently shaken with 0.5 liter of fresh ether, and finally, with caution, 1 liter of water was added without any further shaking. The upper layer plus the main ethereal solution (see above) was washed alkali-free, dried with sodium sulfate, and concentrated as far as possible at not over 40°, under diminished pressure, while a slow stream of CO_2 was bubbled through. In order to eliminate the last traces of the ether, it is advisable to add some petroleum ether (b.p. 70°) and to repeat the evaporation.

The next step was the chromatographic separation of the pigment. The petroleum ether solution of the residue obtained was poured onto a calcium hydroxide column (18 × 3.5 cm.) which was washed with fresh solvent until the following chromatogram was obtained, the zones of which showed blurred borders. Further washing should be avoided, as it would carry the lower layer of pigment into the filtrate. (The figures denote the width of the respective zones.)

40 mm., yellow, heterogeneous: xanthophylls
60 " colorless
2 " very pale yellow: partly carotene (?)
Filtrate: yellowish, differing from carotene but showing a similar spectrum

The two latter fractions were epiphasic; their spectra were in some cases like that of β-carotene, in other cases rather like that of α-carotene, the maxima determined in light petroleum being 482, 452 mμ or 477, 448 mμ.

Neither of these two fractions can, however, be identical with carotene, because their behavior is altered by a new treatment with methanolic alkali. The whole pigment content of the filtrate became hypophasic under this treatment, and an analogous change was observed in the larger part of the 2 mm. zone also when it was eluted in alcohol, transferred into ether, and saponified as mentioned above. As a considerable increase in the adsorbability was also observed, undoubtedly some polyenic esters which are fairly resistant to saponification had been present in the flour.

A further indication of non-identity with carotene was furnished by the so called mixed chromatogram. A solution of pure β-carotene (from red pepper) was added to the saponified fraction and the solution was passed through the Tswett column, whereupon the added carotene was more easily adsorbed, while the flour pigment passed into the filtrate. This flour pigment fraction collected from 60 kilos of flour could not be crystallized. In no case can the possible carotene content exceed 0.01 mg. per kilo of flour.

Xanthophyll—The 40 mm. zone was eluted with alcohol and this solution was mixed with petroleum ether. Sufficient (but no more) water was then cautiously added just to remove the pigment to the upper layer. Too much water would transfer an unusually large amount of the colorless impurities as well. The separated and dried pigment solution was adsorbed on a calcium carbonate column (18 × 4.5 cm.) prepared from a mixture of 1 part of calcium carbonate precip. and 3 parts of calcium carbonate leviss. It is advantageous to develop the chromatogram by means of a benzene-petroleum ether mixture (1:20) and finally to eliminate from the column traces of benzene by washing it with petroleum ether. The developed column showed the following zones.

70	mm.,	colorless	
2	"	brownish yellow	(neoluteins, in light petroleum 473, 444 mμ; when treated with traces of iodine, they are partially converted into lutein)
0.5	"	yellow	
5	"	colorless	
10	"	yellow: xanthophyll (lutein), in light petroleum 477.5, 447 mμ	

The pigmented layers were eluted with alcohol, transferred into light petroleum, and measured with the photometer. The values obtained vary between 1 and 2.5 mg. of xanthophyll per kilo of flour. As xanthophyll is practically the only carotenoid present, for the purpose of an approximate estimation of the whole polyene content it is satisfactory to exhaust 100 gm. of the flour with light petroleum, to concentrate the solution to 100 cc., and to measure the extract photometrically after it has been cleared up by standing.

Isolation of Xanthophyll (Lutein)

We extracted 60 kilos of flour in 5 kilo portions with ether. The first 5 liters of each percolate were worked up directly, as described below, while the following pale solution was used for the extraction of the next 5 kilo portion. The whole liquid was concentrated under a vacuum from 60 to 15 liters. Further concentration was prevented by frothing, which was rapidly getting worse. The saponification with methanolic KOH and the transfer of the coloring matter into ether were carried out as described in the foregoing section, whereupon the solution (3 liters) was washed alkali-free, dried, and evaporated under diminished pressure in a slow stream of CO_2. It was found advisable to dissolve the resulting dark oil in petroleum ether and to evaporate the liquid again.

The chromatogram, obtained on calcium hydroxide (20 × 6 cm.), showed a comparatively well fixed, broad, blurred, but on the whole apparently homogeneous zone, while it was easy to wash the traces of other pigments into the filtrate. The xanthophyll was eluted with alcohol and cautiously transferred into light petroleum with as little water as possible. Finally the whole adsorption procedure was repeated twice more. In the course of such operations it is noticed that the chromatogram gradually improves; *i.e.*, the last column shows a sharply bordered main zone. When this point is reached, the main bulk of the sterols is eliminated and may be easily crystallized from the filtrates.

Nevertheless, further purifying processes are necessary. We eluted the pigment with alcohol and carried out three successive chromatographic separations by using petroleum ether and calcium carbonate. Finally the column was washed with a mixture of benzene and petroleum ether (1:20), and the colored layer was

eluted and transferred into benzene-light petroleum, which was dried and completely evaporated. It left a partially crystallized residue behind, which was dissolved in very little benzene and mixed with light petroleum. There appeared microscopic yellow needles, wart-like grouped, and typical for xanthophyll crystallized in the way described. These were filtered and rapidly dried at 60° under 0.1 mm. pressure.

Yield, 15 mg. of pure lutein, m. p. 193° (corrected). Calculated for $C_{40}H_{56}O_2$, C 84.44, H 9.93; found, C 84.34, H 9.97. Absorption maxima in carbon disulfide, 507.5, 474.5 mμ; in benzene, 489, 457 mμ; in light petroleum, 477, 447 mμ; in alcohol, 477.5, 446.5 mμ. A mixture of a sample of the isolated pigment with lutein (xanthophyll) was inseparable in the Tswett column.

SUMMARY

1. Unbleached wheat flour from southern Hungary contains no more than 0.01 mg. of carotene per kilo, if any. Since it is free also from cryptoxanthin, it is valueless as a provitamin A source.

2. Practically the only polyene occurring in the flours investigated is xanthophyll (lutein). Some of the other pigments are isomerization products (10), while the nature of the others is unknown.

3. Through repeated application of the chromatographic method, 15 mg. of pure xanthophyll crystals have been isolated from 60 kilos of flour.

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DETERMINATION OF SERUM IRON AND PSEUDOHEMO-
GLOBIN IRON WITH o-PHENANTHROLINE

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In a recent publication (1) we reported results obtained by the
use of a new analytical procedure for iron in blood plasma. This
method, which we find to be more sensitive than those which we
have described previously (2–4), depends upon the photoelectric
measurement of the red color of the complex formed by ferrous
iron with o-phenanthroline. This reaction was first applied, as
far as we know, to the determination of small amounts of iron
by Saywell and Cunningham (5). Different aspects of the reac-
tion have been studied by Hummel and Willard (6), Fortune and
Mellon (7), Thiel, Heinrich, and van Hengel (8), and others.
Heilmeyer and Plötner (9) were the first to apply o-phenanthroline
to the measurement of iron in blood serum.

Reagents—

1. Hydrochloric acid, 1.2 per cent solution in water.
2. Hydrochloric acid, 0.3 per cent solution in water.
3. Trichloroacetic acid, 20 per cent solution in water.
4. Sodium acetate, saturated solution in water.
5. 2 M acetate buffer, pH 4.5, prepared by mixing 90 volumes of
2 M sodium acetate and 110 volumes of 2 M acetic acid.
6. Hydrazine sulfate, 1 per cent solution in 2 M acetate buffer,
pH 4.5; this solution must be freshly prepared daily.
7. o-Phenanthroline monohydrate, 0.1 per cent solution in
water; the preparation may be warmed to accelerate solution.
It should be rejected if any color appears.
8. A blank solution prepared by mixing 1 volume of Reagent
1, 1 volume of Reagent 3, and 2 volumes of water. The blank

solution must be changed every time new hydrochloric acid or trichloroacetic acid is prepared.

Procedure

Measure 2 cc. of blood serum or plasma into a short, stout, rimless test-tube (not a conical centrifuge tube, on account of the later difficulty in removing the precipitate). Add 1 cc. of 1.2 per cent hydrochloric acid. Keep stoppered at 37° for at least 1 hour. Cool to room temperature and add 1 cc. of 20 per cent trichloroacetic acid; mix cautiously but thoroughly and let stand 1 hour at room temperature. Cover the tube with tinfoil and centrifuge (3000 R.P.M.) for 15 minutes.

Into a standardized tube, as used with the Evelyn colorimeter, measure 2 cc. of the clear supernatant fluid, 0.5 cc. of saturated sodium acetate solution, 0.5 cc. of the buffered 1 per cent hydrazine sulfate solution, and 0.5 cc. of 0.1 per cent o-phenanthroline monohydrate solution. Prepare a blank Evelyn tube by adding the same reagents to 2 cc. of the blank solution in place of the supernatant fluid.

Allow the blank and the test solution to stand stoppered at room temperature for at least an hour. Then add 2.5 cc. of water to each tube, mix, and read in the Evelyn colorimeter (macro-) using the 6 cc. aperture, Filter 490, and setting the blank at 100. Calculate the iron content of the supernatant fluid used by the usual formula, $100L/K_2$ = mg. of Fe per 100 cc., where K_2 as determined by us is 114.6; to obtain the iron content of the original plasma or serum this value is doubled. The necessary modifications to adapt this method to other types of photoelectric colorimeters will be evident.

Testing of Method—In preliminary experiments with standard ferrous solutions we verified the conformity with Beer's law claimed by others (7, 9) and determined the value for K_2 given above. For a further check on the reproducibility of the color and in particular on the completeness of reduction, a few tests were carried out in which the iron was taken in the ferric state, as ferric ammonium sulfate. The results, in mg. per 100 cc., are as follows:

Ferric iron taken	0.05	0.10	0.15	0.20
Result of analysis	0.051	0.102	0.153	0.197

Table I gives the results of the analysis of samples of horse serum to which had been added measured amounts of ferric iron.

Comment on Method—The preliminary incubation with dilute hydrochloric acid was introduced by Barkan (2, 3, 10) in previously published methods and has been found necessary in all procedures in which iron is to be measured in a protein-free filtrate or ultrafiltrate of blood plasma or serum. After such incubation, trichloroacetic acid filtrates may be prepared and used without the loss of iron mentioned by Fowweather (11) and others. The necessity of such incubation has been noted also by Heilmeyer and Plötner (9) and by Moore, Minnich, and Welch (12). In our experience, the minimal time for incubation with dilute hydro-

TABLE I
Recovery of Ferric Iron Added to Horse Serum
The horse serum contained 0.086 mg. of iron per 100 cc.

Ferric iron added	Calculated result	Analytical result	Error
mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*
0.041	0.127	0.121	−4.7
0.082	0.168	0.169	+0.6
0.082	0.168	0.164	−2.4
0.164	0.250	0.249	−0.4
0.164	0.250	0.260	+4.0

chloric acid is 1 hour at 37°, although we customarily let incubation continue overnight. After the addition of the trichloroacetic acid, another hour should elapse before the precipitated protein is separated in the centrifuge. To obtain a clear solution both the waiting period of 1 hour and the full 15 minutes of centrifugation are necessary.

Traces of hemoglobin in the serum or plasma will not introduce error in this procedure. Hemoglobin is precipitated with trichloroacetic acid. The iron of pseudohemoglobin (about 4 per cent of the hemoglobin iron) is split off by the incubation and appears in the final result. This would be of significance only in grossly hemolyzed sera. A quantitative discussion of this point is given by Heilmeyer and Plötner (9).

For the reduction of the iron and the subsequent reactions the acidity of the trichloroacetic acid filtrate must be decreased. The

addition of a saturated solution of sodium acetate in the amount found necessary from a series of preliminary experiments adjusts the pH of the filtrate to about 4.5 (Hummel and Willard (6)).

The question of the choice of a reducing substance has been discussed by several authors already cited (5-8). Following a suggestion by Snell and Snell (13) we used as our reducing substance a freshly prepared 1 per cent solution of hydrazine sulfate in an acetate buffer at pH 4.5. We recommend fresh preparation of this solution daily, since it was found that its rate of decomposition was quite unpredictable. Measurement of the decrease in hydrazine concentration after 24 hours, by means of the Szebellédy (14) titration, revealed losses varying between 0.25 and 12 per cent.

For complete reduction and color development 1 hour should be allowed after addition of hydrazine and *o*-phenanthroline. The 0.1 per cent solution of *o*-phenanthroline monohydrate can be used as long as it remains colorless, which is usually several weeks provided it is kept in a cool part of the laboratory.

The stability of the red ferrous-phenanthroline complex in slightly acidic solutions, even when exposed to light or to ultraviolet radiation, has been proved by several investigators (5-7, 9). The color remains absolutely unchanged, as shown by the reading of the photoelectric colorimeter, for at least 8 days. To obtain this stability, the hydrazine solution must be fresh; old hydrazine solutions may contain hydrogen peroxide (Gilbert (15), Schales (16)) with resultant oxidation and loss of color of the ferrous complex.

The absorption spectrum of the ferrous-phenanthroline complex has been thoroughly studied (5-7, 9). There is an absorption band between 480 and 520 mμ with its maximum at 509 mμ. For analytical work we have selected the 490 mμ filter of the Evelyn (17) photoelectric colorimeter.

All who have ever done analytical work with small amounts of iron recognize the ubiquity of this element and the necessity of scrupulous precautions against contamination. Even though a blank is used, its iron content should be kept as low as possible. This involves the use of the best analytical grade of chemicals, and the preliminary testing of new lots of reagents. We have not been able to locate a commercial source of trichloroacetic acid

which is entirely free from iron. Reagent grades of the other chemicals have been iron-free as far as our test is concerned. The use of stainless steel needles did not introduce any observable error.

In obtaining plasma for analysis we have used liquoid "Roche" (described by the manufacturers as a sodium polyanethole sulfonate) as the anticoagulant. We have not tried citrate or oxalate. According to Fortune and Mellon (7) citrate does not interfere with the color reaction; oxalate has been shown by them to interfere at pH 6 or less. The interference from phosphate ion in serum or plasma is less than the experimental error.

In connection with some of our earlier work and in particular our recent article (1) dealing with the passage of iron from the red blood cells into the plasma, our experience with normal plasma may be of some interest, since most published data refer to serum. In eighteen normal young men (liquoid as anticoagulant; blood taken with nickel needles; plasma incubated with HCl overnight at 37°) the average plasma iron was 0.113 mg. per 100 cc., with 0.168 as the highest and 0.056 as the lowest value.[1]

Determination of Pseudohemoglobin Iron—The method described above may be used in place of the methods previously described by Barkan (10, 3) for the determination of pseudohemoglobin (18) iron ("easily split off" blood iron). Defibrinated blood, filtered through glass wool, or blood containing hirudin or liquoid, is first diluted with 4 volumes of distilled water. To 10 cc. of this diluted blood, after oxygenation by shaking in air, are added 5 cc. of 1.2 per cent hydrochloric acid. This is then incubated 16 to 24 hours at 37°.

After cooling to room temperature, 5 cc. of 20 per cent trichloroacetic acid are added, and the mixture, after thorough agitation, is allowed to stand 1 hour at room temperature. It is then filtered (Carl Schleicher and Schüll No. 589[1], 9 cm.).

The filtrate is then treated exactly as is the filtrate in the analysis of serum; Evelyn tubes and the same blank solution as previously described are used. The result is multiplied by 10 instead of 2, on account of the original 1:5 dilution of the blood.

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[1] In the article cited (1) attention should be called to two misplaced decimal points in Table I: the iron values for Specimen 10 should read 0.098 and 0.137.

It is often more convenient to use samples of filtrate of less than 2 cc., made up to 2 cc. volume with the blank solution, due account being taken of this extra dilution in the calculations.

SUMMARY

A method for the measurement of the total iron in serum or plasma, or of the pseudohemoglobin iron of whole blood, is described. It involves incubation with dilute hydrochloric acid, reduction of the iron to the ferrous state with hydrazine, and the measurement in a photoelectric colorimeter of the ferrous complex of o-phenanthroline.

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ACID-SOLUBLE PHOSPHORUS COMPOUNDS AND LACTIC ACID IN THE BRAIN*

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Little is known at present concerning the acid-soluble phosphorus compounds in the brain. Kerr and coworkers studied the inorganic phosphate and phosphocreatine, after freezing the brain *in situ* (9–11). Others have analyzed fresh brain tissue for phosphocreatine (7, 15) and for hexose monophosphate (15, 18).

Various methods for the fractionation and determination of acid-soluble phosphorus compounds in tissues have been described, but most of these suffer from the disadvantage that the pyrophosphate is not freed from hexose phosphates and triose phosphates before its hydrolysis. Since some of these esters, as well as pyrophosphate, are hydrolyzed at least partially in 1 N hydrochloric acid during 7 minutes at 100° (3), the pyrophosphate values obtained are not reliable.

Eggleton and Eggleton (2) have described a procedure in which the pyrophosphate fraction is separated from hexose phosphates and triose phosphates by precipitation of the pyrophosphate with barium oxide. This method has the disadvantage that barium sulfate is formed later, after addition of sulfuric acid, in several of the fractions. Barium sulfate occludes phosphate, and the writer has found that in the determination of small amounts of phosphate this occlusion is large enough to introduce an appreciable error.

In the method to be described, the acid-soluble phosphorus compounds are fractionated by a procedure resembling that of

Eggleton and Eggleton, except that calcium hydroxide is substituted for barium oxide as a precipitant. The methods of Fiske and Subbarow are used for the determination of phosphorus in the various fractions (4, 5). Lactic acid may be determined on the same sample by the method of Miller and Muntz (14). The entire analysis is carried out in duplicate on a sample of brain tissue weighing approximately 1 gm.

Methods

Solutions—The preparation of the solutions required for the determination of phosphates has been described by Fiske and Subbarow (4). The only additional solution required is calcium trichloroacetate, prepared by neutralizing 5 per cent trichloroacetic acid with finely ground calcium hydroxide and filtering. The solution should be slightly alkaline to phenolphthalein.

Preparation of Tissue Extract—The brain tissue, about 1 gm. of which.is necessary, is obtained after being frozen *in situ* with liquid air (9, 17). It is crushed, finely ground in a chilled mortar, and added to 12 cc. of 5 per cent trichloroacetic acid at 0° in a tared glass-stoppered flask. This is best done in a cold room. After vigorous shaking, the flask is weighed. 15 minutes are allowed for extraction, with intermittent shaking; then the solution is filtered.

Fractionation of Phosphates—Two 4 cc. aliquots are transferred to graduated centrifuge tubes. To each are added a drop of phenolphthalein and a slight excess of finely ground calcium hydroxide; the contents are stirred with a small glass rod which is subsequently rinsed and removed. After neutralization of the acid, cooling is no longer required. The tube is centrifuged after 10 minutes, and the supernatant fluid decanted into another graduated centrifuge tube. The precipitate is washed with 2 cc. of calcium trichloroacetate, centrifuged, and the washings added to the solution (Fraction A). The precipitate (Fraction B) is dissolved in 4 drops of 5 N hydrochloric acid. Both fractions are diluted to 8 cc.

Determination of Phosphates—Inorganic orthophosphate is determined on Fraction B, and phosphocreatine on Fraction A, by the methods of Fiske and Subbarow (4, 5); 4 cc. aliquots and a final volume of 5 cc. are used.

For the determination of pyrophosphate, a 4 cc. aliquot of Fraction B is mixed with 1 cc. of 5 N hydrochloric acid and heated for 7 minutes in a boiling water bath. After cooling, 3 cc. of water and 1 cc. of 2.5 per cent ammonium molybdate are added, followed by 0.4 cc. of the aminonaphtholsulfonic acid reducing agent and water to a volume of 10 cc., and the color is compared with that of the standard. From the value obtained, the inorganic orthophosphate phosphorus is subtracted to give the pyrophosphate phosphorus present.

Total phosphorus is determined on a 4 cc. aliquot of Fraction A by the method of Fiske and Subbarow (4). It is advantageous to evaporate in a water or steam bath before the digestion, to avoid bumping. After the digestion and addition of water, the solution is heated for a few minutes to change any pyrophosphate to orthophosphate. A final volume of 10 cc. is used. From the total phosphorus present in Fraction A, the phosphocreatine phosphorus is subtracted to give the "hexose phosphate" phosphorus.

In the calculations, whole brain is considered 80 per cent water, cortex 84 per cent water.

Lactic Acid—This may be determined on the trichloroacetic acid filtrate by the method of Miller and Muntz (14). In the experiments described here, the amounts of solution and reagents used by these workers were doubled, and the readings were made with a Duboscq colorimeter equipped with micro cups. The standard solutions used were prepared from zinc lactate (6), and were made slightly acid with sulfuric acid. No blank corrections are necessary. In unpublished experiments it has been found that the amount of pyruvic acid in the brain is very small; hence there is no danger of interference from this source, and the 5 minute heating period may be used.

It was found that the method of Miller and Muntz may also be used with zinc hydroxide filtrates of brain tissue. This is advantageous if lactic acid alone is to be determined, since the filtrates may be prepared at room temperature. Trichloroacetic acid filtrates of brain tissue when prepared at room temperature are turbid, containing organic (lipid ?) material which interferes with the analysis, although no such difficulty is encountered when the filtrates are prepared at 0°.

Block and Bolling (1) have found that under certain conditions the color-producing reaction employed in this determination requires the presence of a trace of lead in the sulfuric acid used. In this laboratory, two brands of C.P. sulfuric acid have been tried and found to be satisfactory. The lead contents were not determined. The addition of traces of lead did not increase the amount of color formed.

DISCUSSION

This modification of previous methods was devised in order to afford a satisfactory means of separating the pyrophosphate fraction from hexose phosphates and triose phosphates, the calcium salts of which are too soluble to be precipitated under the conditions used (5, 13, 16, 19). Fiske and Subbarow (5) have previously separated inorganic phosphate from phosphocreatine by essentially the procedure employed here. It remained to be shown that the precipitation of the pyrophosphate fraction is quantitatively satisfactory. This was done by the following experiment.

A trichloroacetic acid extract of cat brain was prepared. Four 4 cc. aliquots were taken, and the precipitation was carried out as described above. Two of the precipitates were used for the determination of inorganic phosphate and pyrophosphate. Each of the other two was redissolved in 4 cc. of 5 per cent trichloroacetic acid and reprecipitated with calcium hydroxide, and the inorganic phosphate and pyrophosphate then determined. The single precipitation gave the following results, in mg. of phosphorus per 100 gm. of tissue: inorganic phosphate 12.2, 11.9; pyrophosphate 11.5, 11.6. The double precipitation gave for inorganic phosphate 12.0, 11.6 mg.; for pyrophosphate 12.0, 11.3 mg. No phosphorus appeared in the combined filtrate and washings from the second precipitation after 7 minutes at 100° in 1 N hydrochloric acid. It is evident that the precipitation of the pyrophosphate fraction, as well as that of the inorganic phosphate, is complete.

The pyrophosphate fraction is thought to consist almost entirely of adenylpyrophosphate. In the 7 minute hydrolysis, two of the three phosphate groups are split off. In two experiments on cats and five on mice, the total phosphorus in Fraction B was determined. It was found that after subtraction of the inorganic and

pyrophosphate fractions the remaining phosphorus was equal to half the pyrophosphate phosphorus, within the limit of error of the determinations.

The fraction labeled "hexose phosphate" phosphorus includes hexose-6-monophosphate (the Robison-Embden ester) and, if present, hexose diphosphate and triose phosphates, and possibly small amounts of other compounds as well. In four experiments, the part of this fraction hydrolyzing in 7 minutes in 1 N hydrochloric acid at 100° was determined. The hydrolyzable portion varied from 28 to 46 per cent of the "hexose phosphate" phosphorus.

Results

The acid-soluble phosphorus compounds and lactic acid of mouse brain are given in Table I.[1] The mice were frozen in liquid air, as described previously (17). Three brains were usually combined for an analysis. The values given are averages of duplicate determinations. Anesthetized mice were frozen 30 minutes after induction of anesthesia.

In removing the brain from a frozen mouse, great care must be taken to avoid including with it any small fragments of bone which would vitiate the determination of inorganic phosphate without appreciably affecting the other fractions. In three of the analyses given in Table I, the abnormally high values for inorganic phosphate are probably due to failure in this respect, and have been omitted from the averages. In the decapitated mice this difficulty was absent because the brains were removed before being frozen.

The values found for inorganic phosphate and phosphocreatine are in agreement with those of Kerr and coworkers (9–11). The lactic acid values agree with those found previously by a different method (17); the observation of a significant decrease during barbiturate anesthesia is confirmed.

It is evident that during barbiturate anesthesia the phosphocreatine is significantly increased, while the inorganic phosphate is correspondingly decreased.

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[1] On animals deprived of food for 12 hours before the experiment results were obtained like those on animals not fasted.

After decapitation, a large increase in inorganic phosphate occurs. This is partly due to the almost complete breakdown of phosphocreatine and the partial breakdown of adenylpyrophosphate, but is too large to be completely accounted for by these

TABLE I

Acid-Soluble Phosphorus Compounds and Lactic Acid in Mouse Brain

	P per 100 gm. brain				Lactic acid per 100 gm. brain
	Inorganic phosphate	Phosphocreatine	Pyrophosphate	Hexose phosphate	
	mg.	mg.	mg.	mg.	mg.
Normal (3).................	23.7*	9.8	18.6	18.7	19.6
" (3).................	14.8	10.5	14.6	24.8	19.1
" (3).................	17.8	9.6	15.2	20.2	18.1
" (3).................	17.6	10.7	19.0	23.5	18.0
" (3).................	17.5	9.7	18.5	25.1	17.9
Average.................	16.9	10.1	17.2	22.5	18.5
Under dial (3).................	13.0	13.6	15.5	17.3	7.1
" " (3).................	16.7*	14.2	15.9	19.4	5.5
" " (3).................	20.2*	13.2	19.0	21.4	3.4
" " (3).................	13.5	13.5	19.9	24.1	3.8
Average.................	13.3	13.6	17.6	20.6	5.0
Under nembutal (3).................	11.2	14.0	16.2	22.7	9.8
" " (3).................	13.7	13.5	21.6	21.7	9.1
Average.................	12.5	13.8	18.9	22.2	9.5
After decapitation. 5 min. (3)	47.3	2.3	9.3	23.5	98
" " 15 " (2)......	49.0	4.1	7.0	9.7	110
" " 15 " (3).... .	44.5	2.5	·12.6	22.0	108
" " 30 " (3)......	49.6	1.8	10.1	17.7	101
Average.................	47.6	2.7	9.8	18.2	104

The figures in parentheses represent the number of mice.

* Omitted from the average; probably a particle of bone in the sample.

changes. The possibility of phospholipid breakdown is at once evident. Gerard and Wallen (8) found evidence for such a change over a period of time in nerve, and King (12) showed that brain and other tissues contain a lecithinase with optimum pH at 7.5.

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The observed postmortem changes took place during the first 5 minutes after decapitation, with little additional change during a subsequent 25 minute period. It seems possible that the enzyme action is retarded by the high acidity which develops in the brain soon after death. Studies of the pH of the cortex carried out in this laboratory have shown that the pH on the surface drops to at least as low as 6.0 at death (unpublished observations).

In three experiments of this decapitation series the "hexose phosphate" fraction was found to be within normal limits, while in one it was below normal. Tschalissow (18) found amounts of hexose monophosphate corresponding to 7.1 to 10.5 mg. of P per 100 gm. in fresh dog brain, and observed that the amount present decreased only slowly during a period of 2 hours.

SUMMARY

1. Methods are described for the determination of inorganic phosphate, phosphocreatine, adenylpyrophosphate, "hexose phosphate," and lactic acid, in duplicate, on a 1 gm. sample of brain tissue.

2. In brains of normal mice, the following average values were found, expressed in mg. of phosphorus per 100 gm., inorganic phosphate 16.9, phosphocreatine 10.1, adenylpyrophosphate 17.2, "hexose phosphate" 22.5.

3. During barbiturate anesthesia, brain phosphocreatine is increased and inorganic phosphate is correspondingly decreased.

4. The previous finding of a decrease in brain lactic acid during barbiturate anesthesia has been confirmed.

5. After decapitation, brain inorganic phosphate increases at the expense of phosphocreatine and adenylpyrophosphate, and possibly of phospholipids also. Lactic acid shows a large increase. The observed changes occur during the first 5 minutes after decapitation, with little further change during a subsequent period of 25 minutes.

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A NOTE ON THE CONVERSION OF DIIODOTYROSINE INTO THYROXINE

By PAUL BLOCK, Jr.

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The physiological implications of the conversion of diiodotyrosine in part into thyroxine *in vitro*, reported recently (1), are such that it seemed to be of interest to observe the behavior under similar conditions of diiodotyrosine prepared from synthetic tyrosine rather than from tyrosine isolated in the usual way from naturally occurring proteins. The yield of thyroxine reported was so small (20 mg. from 20 gm. of diiodotyrosine) that if an impurity played a part in this reaction a minute quantity would be sufficient to give the reported result. The use of completely synthesized diiodotyrosine seemed to be the most feasible way of insuring the absence of any traces of natural impurities.

The novelty of the chemistry involved in the formation of thyroxine when a solution of diiodotyrosine at slightly elevated pH is allowed to stand at 37° for 2 weeks and the following observation by Ludwig and von Mutzenbecher (2) seemed to indicate a necessity for the repetition of this experiment with absolutely pure diiodotyrosine. These authors reported that they were able to isolate more thyroxine from iodinated casein and from iodinated serum proteins than from iodinated silk fibroin, despite the fact that fibroin contains about twice as much tyrosine. This unexplained fact gives rise to the suspicion that some factor other than tyrosine is playing a part in the reaction, but that such is not the case the following experiment purports to show.

14 gm. of *dl*-diiodotyrosine prepared by iodination of tyrosine synthesized by the method of Harington and McCartney (3) was dissolved in 1 equivalent of 0.1 N NaOH and sufficient 1 N NaOH was added dropwise to bring the pH of the solution to 8.8. The solution was maintained at 37° in a water bath for 14 days. It

was then acidified, the precipitate immediately centrifuged, dissolved in 2 N NaOH, and extracted with butyl alcohol precisely as described by von Mutzenbecher in the case of diiodo-*l*-tyrosine (1).

The residue from the evaporation of the butyl alcohol was dissolved in boiling 0.1 N K_2CO_3 solution; there deposited on cooling a heavy white precipitate resembling in every way the potassium salt of thyroxine.

Addition of a few drops of acetic acid to the solution of the salt in hot 80 per cent alcohol gave 13.5 mg. of a heavy white precipitate with the characteristic crystalline structure of thyroxine: spherules, rosettes, and sheaves.

For analysis the crystallization was repeated.

Analysis—$C_{15}H_{11}O_4NI_4$. Calculated, I 65.38; found, 65.34

The sample melted with decomposition at 233° when heated at the rate of 6° per minute from 190°.

In view of this result it must be concluded that thyroxine is indeed formed directly from diiodotyrosine in mildly alkaline solution at slightly elevated temperatures.

SUMMARY

The conversion of diiodotyrosine into thyroxine *in vitro* has been confirmed, with a completely synthetic diiodotyrosine as starting material.

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CHEMICAL DETERMINATION OF NICOTINIC ACID: INHIBITORY EFFECT OF CYANOGEN BROMIDE UPON THE ANILINE SIDE REACTIONS*

By DANIEL MELNICK† AND HENRY FIELD, JR.

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A paper (1) recently appeared from our laboratory describing a method for the chemical determination of nicotinic acid and related compounds based upon the reaction of nicotinic acid with cyanogen bromide and aniline.[1] Use was made of preferential charcoal adsorption for the decolorization of the test solutions with no concomitant loss of nicotinic acid. The photometric density of the blank due to some color remaining after the charcoal treatment was evaluated by a procedure involving simple dilution of the test solution with an alcoholic buffer reagent. The use of the buffer solution was necessary; dilution with water and alcohol alone yielded, in many cases, too high a value for the blank, because some compounds produced by the hydrolysis, such as cystine and tyrosine, are insoluble at pH 7 but soluble at the acid pH of the chemical reaction.

While our paper (1) was in press, the report by Harris and Raymond (3) appeared, advocating the addition of the base to the blank test. The base, *p*-aminoacetophenone in their method,

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[1] For the measurement of the final yellow color an Evelyn photoelectric colorimeter was used with Filter 420. The design and principle of operation of this instrument have been described by Evelyn (2). For a more complete description, including the significance of the terms used in the present study, the reader is referred to "Evelyn photoelectric colorimeter, Notes on operation" (1939), Rubicon Company, Philadelphia.

TABLE I

Comparison of Values for Dilution and Aniline Blanks in Chemical Determination of Nicotinic Acid; Inhibitory Effect of Cyanogen Bromide upon Aniline Side Reactions

Sample	Preparation	Amount used*	Maximal values,† galvanometer readings‡			Galvanometer readings 16 hrs. later‡			Nicotinic acid content§	
			Dilution blank	Aniline blank	Test with CNBr and aniline	Dilution blank	Aniline blank	Test with CNBr and aniline	With dilution blank	With aniline blank
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
		mg.							mg. per gm.	mg. per gm.
A	Liver extract powder	100	86^3	79^3	45^1	87^3	65^3	75^3	1.08	0.94
B	20% alcoholic liver extract	200	87	80^1	35^3	89^3	61	71	0.72	0.65
C	Dehydrated liver and yeast concentrate	160	78	68^3	25^1	85^3	59^1	66^1	1.19	1.06
D	Dried yeast powder	200	90^2	82^3	55^3	94^3	70^3	81^3	0.43	0.35
E	Yeast extract paste	100	92	86^3	33^3	93^3	67^2	74^1	1.56	1.47
F	" " powder	50	94^3	91^1	47^3	96^2	70^3	83^3	2.40	2.26
G‖	Sweetened, concentrated aqueous extract of yeast	200	89^3	80^3	62^2	92^3	67^3	83^3	0.31	0.22
H‖	Aqueous concentrate of rice polish extract	25	93^3	91^3	69^1	94^3	61^2	91	1.96	1.81
I‖	Wheat germ powder	300	76^3	63^1	62^3	84^3	34	79	0.12	0.0

	cc.							mg. per 24 hrs. 3.0 (mg. per cent)	mg. per 24 hrs. 1.0 (mg. per cent)
J|| Human urine (24 hr. sample = 730 cc.)	15.2	61[3]	51[3]	42[3]	66[2]	42[3]	51[1]	3.0	1.0
K|| Human saliva	30	94[2]	92[3]	92[1]	90[1]	70[3]	96[3]	0.0	0.0
L|| " oxalated plasma	10	41	30[1]	35[3]	44[3]	35[3]	40	0.22	0.0
M|| " blood	10	60[3]	58[3]	39[3]	69[2]	58	64	0.69	0.64
N|| Cow's milk	10	63[3]	52[3]	48[3]	63[3]	30[4]	55[3]	0.44	0.12
10 γ nicotinic acid in 3 cc. H2O-C2H5OH (2:1)		100	100	58[1]	100	100	88[3]	γ 10	γ 10
Center setting		70	72[1]	88[1]	70	72[1]	89[3]		

* In all cases the colorimetric tests were conducted with one-tenth aliquots of the starting materials.

† The maximal color intensities, in tests for nicotinic acid, were obtained from 3 to 10 minutes after the reagents were added. During this period values for both blanks were constant.

‡ These readings have been corrected for the slight deviations from true linearity of the relation between current and deflection.

§ The method for calculating these values has been described elsewhere (1).

|| These materials were not taken from the same samples tested and reported elsewhere (1).

was found to react directly with substances in the hydrolysates to give colors indistinguishable from that obtained in tests for nicotinic acid. We have also found this to be true with aniline. However, our studies indicated that in the presence of cyanogen bromide these interfering side reactions do not occur. Accordingly, we omitted aniline from the blank test. Experimental proof to support the validity of this procedure is here presented.

For convenience, the procedure already reported by us for making the blank correction will be referred to as the "dilution blank," while the other, which includes the addition of the base, will be called the "aniline blank." In the latter case, 6 cc. of 0.03 M H_3PO_4, in addition to the aniline, were added in place of the buffer solution in order to simulate test conditions with respect to pH and titratable acidity.

In Table I are presented the results of the analyses conducted with the same test materials listed in our earlier report (1). In every case aniline was found to react directly with substances in the neutralized alcoholic hydrolysates to give extraneous color (compare the values in Column 4 with those in Column 5). For this reason the figures for the nicotinic acid content of the materials tested were always less when the aniline blanks were used in the calculations. In some cases the differences resulting from the use of these two types of blanks were large (e.g., in the case of Samples G, I, J, L, and N). When the solutions tested for nicotinic acid are allowed to stand, there is a decrease in the amount of yellow pigment formed (i.e., increase in galvanometer readings). Thus, when the colorimetric measurements were made 16 hours after the addition of the reagents, much smaller values were obtained compared with the maximal figures. After this time, however, in every case the values for the aniline blanks were much greater than those for the solutions tested for nicotinic acid although the latter had had the same amount of aniline added originally and still contained an appreciable fraction of the yellow reaction product (compare the values in Column 8 with those in Column 9). Apparently aniline reacts with substances in the hydrolysates to give colors which are not obtained in the presence of the cyanogen bromide reagent.

Additional tests have shown that cyanogen bromide can actually reverse the aniline side reactions even after these had taken

place. By changing the normal order of addition of the reagents so that the base was added first to the test solutions, the side reactions giving rise to extraneous color were obtained. When the cyanogen bromide reagent was then added, the color was no greater than that obtained in the test conducted in the usual routine manner. This was true for both maximal readings and those taken 16 hours after the addition of the reagents.

More crucial experiments involving the use of charcoal adsorption to remove the nicotinic acid from solution have shown that the substances which react directly with aniline are not adsorbed and yield color with the base only in the absence of the cyanogen bromide. 3 gm. quantities of charcoal were added to $\frac{1}{2}$ hour urine samples at pH 7; the mixtures were brought to a boil and filtered. The remainder of the analyses with aliquots of the filtrates followed the procedures described in the earlier report (1). Colorimetric measurements made from 5 to 10 minutes after the addition of the reagents showed that 90 per cent of the nicotinic acid originally present had been removed. The values for the aniline blanks, however, were large and in very great excess of those obtained with the solutions tested for nicotinic acid.

In the course of our studies more than 80 urine samples from eleven normal subjects, receiving nicotinic acid solely from the diet, were analyzed for nicotinic acid. Each specimen was subjected to two types of acid hydrolysis, one of 30 minutes, the other of 5 hours duration. Calculations, with the dilution blank, gave practically the same nicotinic acid values for each sample regardless of the length of the period of hydrolysis. With the aniline blank, the values obtained subsequent to the 30 minute hydrolysis were generally very much less, while those following the hydrolysis of 5 hours duration closely approximated the values obtained with the dilution blank. Apparently, the substances in the hydrolysates which react directly with the base are either volatilized or altered during the long period of hydrolysis, with the result that smaller values for the aniline blanks are obtained. Since the aniline side reactions are not observed in the presence of cyanogen bromide in either case, after short or prolonged hydrolysis, the same values are obtained when the dilution blanks are used in the calculations.

SUMMARY

In the analyses of biological materials aniline was found to react directly with substances in the hydrolysates to give colors indistinguishable from that obtained in tests for nicotinic acid. In the presence of cyanogen bromide these interfering side reactions do not occur. Aniline, therefore, should not be included in the blank tests.

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INDIVIDUAL PHOSPHOLIPIDS IN PLASMA OF RABBITS AFTER FATTY MEALS*

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The present investigation was suggested by the idea that the variations of individual phospholipids in the plasma of animals absorbing large amounts of fats might give an indication of their possible rôle in fat transport.

The literature on the subject has been reviewed recently by Bloor (4). Therefore only the data published by Rubin (9), when our experiments were in an advanced stage, need to be mentioned: a slight increase in total phospholipids with doubtful variations of cephalins was noticed in the plasma of two dogs which were fed fat.

It should also be noted that in hemorrhagic lipemia of rabbits a progressive increase of both lecithins and cephalins has been found, though the increase was generally greater in the lecithin fraction (10).

EXPERIMENTAL

Seven series of experiments have been made on ten female rabbits kept on a standard mixed diet. Each series consisted of five (or six) experiments; Experiment 1 in each series was without oil feeding. Then at intervals of at least 10 days the animals received 10 gm. of olive oil per kilo by stomach tube; and after 15, 30, or 45 hours blood was collected over NaF and centrifuged. One other experiment, either Experiment 4 or 5, was made without oil feeding, also after a lapse of at least 10 days. Because of the moderate amount of blood taken (6 to 12 cc. per animal) and the

considerable intervals between experiments, the interference of hemorrhagic lipemia was certainly avoided.

Procedure

Our procedure is based on that of Kirk (7) with the modifications noted below.[1]

Plasma lipids have been extracted with cold and boiling alcohol and purified with chloroform,[2] as by the senior author in previous work (see especially (1)). The precipitation and resolution of phospholipids into ether-soluble and ether-insoluble fractions were carried on as described by Kirk (7), except that larger amounts of acetone, $MgCl_2$, and ether were used, and that before centrifugation the phospholipid precipitate was placed in a refrigerator overnight. Acetone-soluble lipids, after evaporation of the solvent, were reextracted with dry purified petroleum ether (b.p. <50°), and the extract filtered through asbestos. On the filtrate duplicate determinations with Bloor's oxidimetric procedure (2) were carried out, the conventional factor of 3.75 being used for the calculation of lipids from 0.1 N dichromate. Phosphorus and choline were determined on aliquots of (a) the initial chloroform solution, (b) the ether-soluble, and (c) the ether-insoluble fraction (redissolved in a methanol-chloroform mixture).

Phosphorus was estimated by Tisdall's colorimetric procedure (12), the directions given by Kirk (6) being followed. Phospholipids were calculated with the factor 25 for the phospholipid to phosphorus ratio.

For choline determinations, the samples were hydrolyzed, according to Thannhauser et al. (11), by refluxing with saturated gaseous HCl in methanol, later removed by suction at a temperature below 55°. The residue is dissolved in 5 cc. of water, filtered through asbestos, and the volume of the filtrate noted. Choline

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[1] Preliminary attempts to apply the precipitation of sphingomyelins with Reinecke salt (according to Thannhauser et al. (11)) with subsequent phosphorus determination in the precipitate were not satisfactory, probably because of the small amounts of available material.

[2] The substitution of chloroform for petroleum ether avoids the danger of losses due to the insolubility of partially oxidized phospholipids in petroleum ether. Moreover the solubility of sphingomyelins in petroleum ether is slight (5). However, in chloroform extracts the oxidation of ether-soluble phospholipids often gives too high results compared with phosphorus.

is then precipitated and evaluated according to Roman (8). Though great care was used, especially at the end of the evaporation of the HCl-methanol solution, a small loss of choline could not be avoided. In controls we found the loss almost constant, averaging 0.03 mg., irrespective of the amount of choline; therefore results have been corrected correspondingly throughout. The factor 6.65 was used in the calculation of phospholipids from choline.

In our analytical scheme lecithins may be calculated (a) from the choline in the ether-soluble fraction or (b) from the difference between the choline in the chloroform extract and that in the ether-insoluble fraction; the ether-insoluble phospholipids, (a) from their phosphorus or (b) from their choline content.[3] Only cephalins must be calculated indirectly, from the difference between the total phospholipids and the choline-containing phospholipids in (a) the chloroform extract or (b) the ether-soluble fraction. The possibility of checking the results by duplicate calculations is obviously a great advantage of the procedure outlined above.

<div align="center">DISCUSSION</div>

Table I shows our results, the figures being the averages of the values calculated in the two ways indicated.

Though, in accord with previous findings (3), an alimentary lipemia was only occasionally seen, higher values for lecithins and lower for cephalins with a consequent shift in the lecithin to cephalin ratio were mostly found after oil feeding. Moreover in those oil feeding experiments in which there was a clear increase in total phospholipids, it was essentially or even entirely in the lecithin fraction.[4]

[3] HCl in methanol insures a more complete hydrolysis of sphingomyelins than baryta. Unlike Kirk's results (7), a rather satisfactory agreement between the phosphorus and choline in the ether-insoluble fraction was found in all but three of our experiments, the averages of phospholipids in the other thirty-four being 34.9 per cent as calculated from the phosphorus and 36.1 per cent as calculated from choline.

[4] Because of the intersolubility of lipids, the separation of sphingomyelins by their insolubility in ether may be questioned. In such a case, since the sum of the lecithins and the ether-insoluble phospholipids shows the same qualitative behavior as lecithins alone, we should merely state that after oil feeding there is generally an increase in the choline-containing phospholipids.

TABLE I

Lipid Fractions in Plasma of Rabbits without and after Fatty Meals

The results are given in mg. per 100 cc. of plasma.

Rabbit	Experiment No.	Date	Hrs. after fatty meal	Phospholipids				Acetone-soluble lipids
				Leci-thins	Ceph-alins	Ether-insoluble	Total	
A and B	1	Sept. 16	No oil	44	77	47	168	308
	2	" 30	45	101	44	38	183	427
	3	Oct. 9	15	83	7	41	131	278
	5	Nov. 11	30	41	32	33	106	249
	4	Oct. 26	No oil	54	14	41	109	269
C*	1	Sept. 26	" "	33	49	29	111	516
	2	Oct. 9	30	97	29	34	160	499
	3	" 21	45	36	0	36	72	315
	4	Nov. 6	15	87	0	37	114	375
	5	" 20	No oil	30	7	75	112	466
D	1	Sept. 29	" "	28	25	42	95	201
	2	Oct. 16	30	90	7	40	137	368
	3	" 31	15	69	0	44	113	244
	4	Nov. 16	15	39	0	38	77	219
	6	Dec. 16	45	62	6	18	86	233
	5	Nov. 27	No oil	17	7	45	69	189
E	1	Oct. 17	" "	36	3	35	74	350
	2	" 28	30	44	0	35	79	234
	3	Nov. 8	15	85	23	54	162	477
	5	" 30	45	69	6	58	133	381
	4	" 19	No oil	48	50	24	122	334
F and G	1	" 21	" "	24	52	49	125	270
	2	Dec. 2	15	45	17	37	99	245
	3	" 13	30	79	0	50	129	270
	5	Jan. 20	45	116	0	23	139	429
	4	" 4	No oil	73	0	31	103	326
H and I	1	Dec. 8	" "	45	39	25	109	285
	2	Jan. 4	15	80	0	16	96	244
	3	" 20	30	100	0	21	121	495
	4	Feb. 1	45	103	6	20	129	248
	5	" 20	No oil	64	8	28	100	151
J†	1	Jan. 23	" "	15	17	18	50	93
	2	Feb. 10	15	22	5	19	46	92
	3	" 25	30	50	0	13	63	104

* Oil was given with 1.5 gm. of sodium mono- and diphosphate, 0.1 gm. of choline chloride, and 0.05 gm. of cholamine, and in the controls starch was given with these substances 15 hours before blood collection.

† The series of experiments for Rabbit J was incomplete because of the accidental death of the animal.

Such findings may be regarded as in line with the hypothesis that plasma lecithins and not cephalins or sphingomyelins are involved in the transport of absorbed fatty acids; therefore they also agree with other facts, especially the effectiveness of choline and the ineffectiveness of cholamine in prevention and cure of fatty liver.

However, besides the great variability of individual values in both control and oil feeding experiments and the failure to produce a noticeable alimentary lipemia in most cases, the discrepancy between initial and final controls is rather puzzling, final values obtained on animals to which no oil has been fed for at least 10 days frequently being intermediate between initial values and those after oil feeding. Because of these facts, the possibility that the variations described are not directly correlated with the carrying of absorbed fatty acids in the plasma of rabbits cannot be entirely excluded. Consequently we do not consider our results as conclusive evidence of the rôle played by individual phospholipids in fat transport, but we think that further work is required before the problem will be definitely solved.

SUMMARY

1. By a modification of Kirk's procedure, lecithins, cephalins, and ether-insoluble phospholipids have been estimated in the plasma of rabbits before and after the administration of single large doses of olive oil.

2. Higher values for lecithins and lower for cephalins were mostly obtained after oil feeding. The variations of ether-insoluble phospholipids were generally slighter and irregular.

3. The significance of the above results for the hypothesis that lecithins are the only phospholipids involved in the transport of absorbed fatty acids is discussed.

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THE DISSOCIATION OF CALCIUM AND MAGNESIUM PHOSPHATES

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There is an increasing body of evidence (1–7) tending to show that the salts of divalent cations with organic acids are only incompletely dissociated in aqueous solution. This apparent incomplete dissociation seems also to be true of calcium sulfate (2).

The present paper is concerned with the possibility of such apparent incomplete dissociation of calcium phosphate and of magnesium phosphate. The existence of a soluble complex of calcium, carbonate, and phosphate was postulated by Benjamin and Hess (8) but the unsatisfactory character of their evidence was pointed out by Greenberg and Larson (9). In spite of the reply by Benjamin (10), we believe that the observations of Greenberg and Larson, which have been repeated in this laboratory (11), make it impossible to consider the experiments of Benjamin and Hess as valid evidence of the existence, in solution, of the postulated complex.

From the equation, pH = pK + log (base/acid), it is evident that the addition, to a mixture of an acid and its conjugated base, of the halide of any cation capable of forming a complex with the anion must decrease the pH of the mixture. The effect is that of a lowering of the apparent value of pK and was, in fact, so regarded by Simms (12) and by Shima (13).

The effect is readily measurable and the values obtained may be used for calculation of the degree of association of the ions. We, therefore, determined the pH of various mixtures of phosphoric acid and sodium hydroxide, in the presence of $CaCl_2$, or of $MgCl_2$, and compared the values with those obtained in

similar mixtures containing NaCl or KCl of the same ionic strength.

<div align="center">EXPERIMENTAL</div>

Calcium, magnesium, sodium, and potassium chloride solutions were prepared from good grades of commercial salts. The con-

<div align="center">TABLE I</div>

<div align="center">Titration of 1.868 mм H_3PO_4 in Presence of 11.60 mм NaCl or of 3.869 mм $MgCl_2$</div>

$\mu = 0.0015$.

NaOH added	NaCl		MgCl₂		
	pH	pK₂ H₃PO₄	pH	α	pK MgHPO₄
mм					
1.20	3.32		3.32		
1.60	3.78		3.79		
1.80	4.59		4.50		
2.00	6.01	7.10	5.75	0.026	2.17*
2.20	6.34	7.09	6.10	0.089	2.42
2.40	6.61	7.11	6.38	0.131	2.38
2.60	6.82	7.00	6.59	0.185	2.40
2.80	7.00	6.96	6.80	0.220	2.36
3.00	7.15	7.13	6.98	0.278	2.40
3.20	7.40	6.98	7.15	0.354	2.49
3.40	7.64		7.42	0.400	2.40
3.60	7.99		7.78	0.541	2.67*
3.80	9.30		8.75†		

<div align="center">Average $K_2 = 8.80 \times 10^{-8}$ Average $K = 3.93 \times 10^{-3}$
pK₂ = 7.06   pK = 2.41‡</div>

* Excluded from the average.

† At this pH, the calculated amount of NaOH to be added in NaCl solution is 3.66 ml. or 1.96 equivalents per mole of acid. α is indeterminate. The formula $(B/S_a - R)/2 - R$ gives $\alpha = 0$, but a change of pH to 8.72 makes $\alpha = 1$.

‡ This number is the negative logarithm of the average value of K. This procedure has been followed in all the tables.

centrations of the first two were determined from the chloride content; the sodium and potassium chlorides were weighed as such. Phosphoric acid was prepared from syrupy phosphoric acid and was standardized by precipitation and weighing as $MgNH_4PO_4 \cdot 6H_2O$.

A series of volumetric flasks, each containing the same amount of phosphoric acid, salt, and water, was prepared for each titration. 0.1 N NaOH was then added. After the contents had been diluted to the mark and mixed, the pH was determined with the Coleman glass electrode at room temperature, with a saturated solution of KCl for the liquid junction. In a number of instances, the pH of the mixtures containing Ca or Mg chloride was determined at intervals for 2 hours. No change was observed.

TABLE II

Titration of 1.491 mM H_3PO_4 in Presence of $MgCl_2$

NaOH added	7.737 mM $MgCl_2$, μ = 0.026			15.47 mM $MgCl_2$, μ = 0.049		
	pH	α	pK	pH	α	pK
mM						
1.60	5.68	0.027	1.88*	5.40	0.032	1.77*
1.80	6.12	0.097	2.06	5.89	0.107	1.88
2.00	6.39	0.149	2.05	6.19	0.167	1.85
2.20	6.61	0.216	2.06	6.38	0.259	1.95
2.40	6.82	0.329	2.21	6.60	0.364	2.01
2.60	7.11	0.383	2.18	6.88	0.435	1.99
2.80	7.49	0.474	2.22			

K_2 of H_3PO_4 in NaCl = 1.06×10^{-7} 1.49×10^{-7}

Average $K = \dfrac{[Mg^{++}][HPO_4^{-}]}{[MgHPO_4]} =$

7.47×10^{-3} 1.74×10^{-2}

pK = 2.13   1.93

* Excluded from the average.

In another series of experiments, the hydrogen electrode, at 25°, was employed. In these, the variations in the proportions of phosphoric acid and of calcium and magnesium chloride were greater than in the first series and there were a number of experiments in which mixtures of potassium chloride and calcium chloride or magnesium chloride were used. The titration intervals were greater than in the first series. Mixtures in which a precipitate formed either immediately or within 24 hours were discarded.

The composition of the mixtures and the values for pH obtained are given in Tables I to VI. The ionic strengths are calculated

TABLE III

Titration of 0.747 mM H_3PO_4 in Presence of $CaCl_2$

NaOH	1.515 mM CaCl₂, μ = 0.006			3.788 mM CaCl₂, μ = 0.013			7.576 mM CaCl₂, μ = 0.024		
	pH	α	pK	pH	α	pK	pH	α	pK
mM									
0.80				5.80	0.032	2.21			
0.88	6.35	0.048	2.37	6.22	0.057	2.04			
0.90							6.22	0.094	2.04
0.96	6.64	0.059	2.24	6.49	0.086	2.06	6.39	0.125	2.03
1.01							6.50	0.172	2.11
1.05	6.90	0.066	2.11	6.71	0.133	2.12			
1.06							6.66	0.188	2.16
1.12							6.76	0.244	2.12
1.14				6.91	0.180	2.15			
1.15	7.14	0.076	2.04						
1.17							6.81	0.328	2.27
1.24	7.31	0.165	2.37	7.11	0.252	2.24			

K_2 of H_3PO_4 in NaCl =
7.53 × 10⁻⁸   9.00 × 10⁻⁸   8.69 × 10⁻⁸

Average $K = \dfrac{[Ca^{++}][HPO_4^{-}]}{[CaHPO_4]} =$
6.24 × 10⁻³   7.40 × 10⁻³   7.66 × 10⁻³
pK = 2.20   2.13   2.12

TABLE IV

Titration of 1.488 mM H_3PO_4 in Presence of $MgCl_2$

NaOH	8.33 mM MgCl₂, μ = 0.027			13.3 mM MgCl₂, μ = 0.043		
	pH	α	pK	pH	α	pK
mM						
1.879	6.226	0.137	2.12	6.136	0.141	1.95
2.192	6.558	0.261	2.22	6.459	0.727	2.05
2.506	6.945	0.399	2.26	6.814	0.563	2.15

K_2 of H_3PO_4 in KCl = 1.02 × 10⁻⁷   1.20 × 10⁻⁷

Average $K = \dfrac{[M^{++}][HPO_4^{-}]}{[MHPO_4]} = 6.37 \times 10^{-3}$ 9.10 × 10⁻³

pK = 2.20   2.06

without correction for the complex formed. The effect of this upon the ionic strength and the dissociation of phosphoric acid was negligible. The values for the dissociation constant of phosphoric acid given in Tables I to VI were calculated in the usual manner from the data obtained in solutions containing NaCl or KCl. It will be noted that the values of K_2 are not quite the same, at identical ionic strength, in Tables II and III. However, the values used were, in each case, obtained with the electrometer standardized on the same occasion for mixtures containing NaCl as for those containing CaCl$_2$ or MgCl$_2$. If an error in standardi-

TABLE V

Titration of 0.744 mM H_3PO_4 in Presence of CaCl$_2$

NaOH	8.33 mM CaCl$_2$, μ = 0.027			13.3 mM CaCl$_2$, μ = 0.043		
	pH	α	pK	pH	α	pK
mM						
0.940	6.281	0.136	2.08	6.193	0.125	1.84
1.086	6.573	0.341	2.38	6.566	0.221	1.84
1.253	6.958	0.364	2.16			

K_2 of H_3PO_4 in KCl = 1.02×10^{-7}	1.20×10^{-7}
Average $K = \dfrac{[M^{++}][HPO_4^{--}]}{[MHPO_4]} = 6.47 \times 10^{-3}$	
pK = 2.19	

zation was responsible for a displacement of the titration curve in NaCl solution, there should have been a parallel, and equal, displacement in CaCl$_2$ or MgCl$_2$ solution.

Results

No difference in pH between mixtures containing CaCl$_2$ or MgCl$_2$, and those containing NaCl was observed until after the addition of more than 1 equivalent of alkali per mole of phosphoric acid. Because precipitates appeared in many of the more alkaline mixtures, the complete range of dissociation of the second proton of phosphoric acid could be followed in only one experiment, that with the lowest concentrations of phosphoric acid and of magnesium (Table I). Inspection of the figures shows that the titration in the presence of MgCl$_2$ became nearly the same as in NaCl, as the pH approached 9.0.

TABLE VI

Titrations in Solutions Stabilized at $\mu = 0.2$ by Addition of KCl As Needed

K_2 of H_3PO_4 in KCl = 1.78×10^{-7}.

11.16 mм H_3PO_4

N_a (mм)	13.33 mм MgCl₂			13.33 mм CaCl₂		
	pH	α	pK	pH	α	pK
12.528	5.784	0.029	1.33*			
14.616	6.209	0.111	1.67	5.833	0.014	1.80
16.704	6.569	0.156	1.60			

Average $K = 2.33 \times 10^{-2}$
pK = 1.62

5.58 mм H_3PO_4

N_a (mм)	33.33 mм MgCl₂			13.33 mм CaCl₂			33.33 mм CaCl₂		
	pH	α	pK	pH	α	pK	pH	α	pK
6.264	5.578	0.064	1.52	5.826	0.021	1.18	5.637	0.074	1.62
7.308	6.008	0.185	1.66	6.298	0.065	1.31			
8.352	6.348	0.298	1.67						

Average $K = 2.44 \times 10^{-2}$
pK = 1.61

1.116 mM H₃PO₄

	0.07 mM MgCl₂			13.3 mM MgCl₂			0.07 mM CaCl₂			13.3 mM CaCl₂		
1.253	5.784	0.034	1.73	5.771	0.032	1.42	5.818	0.022	1.55	5.844	0.017	1.07*
1.462	0.332	0.047	1.43	0.189	0.120	1.08	0.350	0.038	1.40	0.286	0.074	1.37
1.670	0.646	0.102	1.60	0.449	0.246	1.88	6.078	0.072	1.41	0.571	0.100	1.52
1.879	7.015	0.102	1.43	0.782	0.304	1.93				0.879	0.258	1.07
Average	Average $K = 2.07 \times 10^{-2}$ pK = 1.53			2.10×10^{-2} 1.08			3.38×10^{-2} 1.47			3.00×10^{-2} 1.52		

	33.33 mM MgCl₂			66.07 mM MgCl₂			33.33 mM CaCl₂			66.07 mM CaCl₂		
1.253	5.504	0.076	1.67	5.380	0.091	1.56	5.583	0.067	1.53	5.519	0.076	1.35
1.462	5.854	0.225	1.80	5.719	0.248	1.78	5.952	0.203	1.75	5.938	0.206	1.44
1.670	0.332	0.308	1.64	6.019	0.403	1.81						
1.879	0.511	0.502	1.92									
1.094	0.797	0.531	1.82									
Average	Average $K = 1.65 \times 10^{-2}$ pK = 1.78			2.03×10^{-2} 1.69			2.37×10^{-2} 1.52					

Average of all pK = 1.65 ±0.16 s.D.

1.50 ±0.17 s.D.

* Excluded from the average.

DISCUSSION

The absence of any effect of $CaCl_2$ or of $MgCl_2$ upon the titration of the first proton of phosphoric acid indicates that there was, in these experiments, no appreciable formation of complexes of Ca^{++}, or of Mg^{++}, with $H_2PO_4^-$.

Because of the formation of precipitates, we were rarely able to continue our observations beyond pH 7.0, and, in only one case, beyond pH 8.0. Only above this pH does the concentration of $PO_4^=$ become significant. Therefore, our results yield no information on the tendency of this ion to associate.[1]

The effects observed must, therefore, be ascribed to association of Ca^{++}, or of Mg^{++}, with $HPO_4^=$. The most probable product is $MHPO_4$. Accordingly, the data obtained have been treated upon this assumption.

The method of calculation resembles that employed by Cannan and Kibrick (2) in the study of complex formation with dibasic acids.

The postulated complex $MHPO_4$ might dissociate into M and HPO_4. The constant for this would be

$$K_{MHPO_4} = \frac{[M^{++}][HPO_4^=]}{[MHPO_4]} \tag{1}$$

However, it might also dissociate into H and MPO_4, for which we may write

$$K_4 = \frac{[H^+][MPO_4^-]}{[MHPO_4]} \tag{2}$$

If S_a = concentration of total phosphate and α = the fraction present as complex,

$$\alpha S_a = [MPO_4^-] + \frac{[MPO_4^-][H^+]}{K_4} \tag{3}$$

from which

$$[MPO_4^-] = \alpha S_a \frac{K_4}{[H^+] + K_4}$$

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[1] If the formation of undissociated $M_3(PO_4)_2$ were appreciable in these experiments, the value of the dissociation constant, $K = [M^{++}]^3 [PO_4^=]^2/[M_3(PO_4)_2]$, would be of the order of 10^{-17}.

If $B' = $ total base (titration $+ H^+$), then, at our range of pH,

$$B' = (1 - \alpha)S_a \left(\frac{[H^+]^2}{[H^+]^2 + H^+ K_2} + \frac{2[H^+]K_2}{[H^+]^2 + H^+ K_2} \right) + 2\alpha S_a + \frac{\alpha S_a \cdot K_4}{[H^+] + K_4}$$

$$= (1 - \alpha)S_a \left(\frac{[H^+] + 2K_2}{[H^+] + K_2} \right) + \alpha S_a \frac{3K_4 + 2[H^+]}{[H^+]K_4}$$

$$= (1 - \alpha)S_a \cdot R + \alpha S_r \cdot V \tag{4}$$

and

$$\alpha = \frac{B'/S_a - R}{V - R} \tag{5}$$

in which

$$R = \frac{[H^+] + 2K_2}{[H^+] + K_2} \quad \text{and} \quad V = \frac{3K_4 + 2[H^+]}{[H^+] + K_4} \tag{6}$$

From Equations 2 and 3

$$MHPO_4 = \frac{[H^+]\alpha S_a}{[H^+] + K_4} \tag{7}$$

When the appropriate values are substituted in Equation 1,

$$K_{MHPO_4} = \frac{[\text{total M} - \alpha S_a]\left(\dfrac{K_2}{[H^+] + K_2} \cdot (1 - \alpha)S_a \right)}{\dfrac{[H^+]\alpha S_a}{[H^+] + K_4}}$$

$$= \frac{1 - \alpha}{\alpha} \cdot \frac{K_2}{[H^+] + K_2} \cdot \frac{[H^+] + K_4}{[H^+]} [\text{total M} - \alpha S_a] \tag{8}$$

It at first appeared reasonable to believe that K_4 would be of the order of 10^{-2} or 10^{-4}. However, substitution of such values in Equation 8 failed to give constant or reasonable values for K_{MHPO_4}. On the other hand, the assumption that K_4 was much lower than $[H^+]$, 10^{-9} or less, gave reasonable and consistent values for K_{MHPO_4}. As K_4 is made smaller with respect to $[H^+]$, the value of V approaches and becomes equal to 2. Accordingly, all subsequent calculations were based upon the assumption that $V = 2$ and that

$$\alpha = \frac{B'/S_a - R}{2 - R} \tag{9}$$

and

$$K_{\text{MHPO}_4} = \frac{1-\alpha}{\alpha} \cdot \frac{K_2}{[\text{H}^+] + K_2} \, [\text{total M} - \alpha S_a] \qquad (10)$$

In these calculations, $[\text{H}^+]$ was taken as equal to $a[\text{H}^+]$. In the value for B$'$, $[\text{H}^+]$ was of significance in only a few instances, so the effect on B$'$ of substituting $[\text{H}^+]$ by $a[\text{H}^+]$ was negligible.

As is apparent from the results presented in Tables I to VI, the assumptions outlined above lead to fairly constant values for the dissociation of the supposed complex. A few of the recorded values diverge considerably, but inspection shows that these were obtained from measurements made under conditions at which α (the fraction of phosphate bound) was small. Such low values of α are encountered at low concentrations of MCl$_2$ or at low pH. Under these conditions, the difference between the amounts of alkali required by the mixtures containing MCl$_2$ and those containing NaCl or KCl is very small. A similar unreliability is to be expected at the upper range of pH, as the titration curves again approach each other. In our experiments, this occurred only once (Table I). It will be noted that pK calculated for pH 7.78 was 2.67, instead of the 2.36 to 2.49 obtained for pH 6.10 to 7.42. At pH 8.75, α became indeterminate.

Table VI shows that concordant values of pK are to be obtained at $\mu = 0.2$, regardless of whether all, or only 10 per cent, of this is furnished by MCl$_2$.

After the experimental work reported in this paper had been completed, it was discovered that Bjerrum and Dahm (14) had obtained a similar, and more marked, effect of AlCl$_3$ upon the apparent dissociation of phosphoric acid. Their results indicated the formation of several types of complex, including Al(H$_2$PO$_4$)$_3$.

At this time, there appeared a paper by Shima (13) in which he reported the effect of KCl, NaCl, MgCl$_2$, and MgSO$_4$ upon the pH of half neutralized 12.5 mM phosphoric acid, as determined by the quinhydrone electrode. By calculation from his data, we obtained the figures shown in the last column of Table VII. The first three figures are rather unreliable, because they are based on differences in pH of only 0.037, 0.055, and 0.075, respectively. Thereafter, the figures are quite in agreement with ours and show the same increasing dissociation with increasing ionic strength.

The equation $pK = 2.50 - 2.15 \sqrt{\mu}$ gives fair agreement with the observed values, whether obtained by Shima with the quinhydrone electrode or by us with glass and hydrogen electrodes.

Physiological Significance—The value of the dissociation constant for $CaHPO_4$ indicates that in plasma, at pH 7.35 and $\mu =$

TABLE VII

Relation of $pK = -Log \dfrac{[M^{++}] [HPO_4^-]}{[MHPO_4]}$ *to Ionic Strength*

	CaHPO$_4$		MgHPO$_4$			Calculated from $pK = 2.50 - 2.15 \sqrt{\mu}$
μ	Glass electrode	Hydrogen electrode	Glass electrode	Hydrogen electrode	Quinhydrone electrode	
0.006	2.20					
0.013	2.13					
0.015			2.41			2.24
0.026	2.12		2.13			2.15
0.027		2.19		2.20		2.15
0.030					1.84*	2.13
0.032					1.57*	2.11
0.034					1.85*	2.10
0.040					1.94	2.07
0.041		1.84				
0.043				2.06		2.05
0.044					2.01	2.05
0.049			1.93			2.02
0.055					1.97	2.00
0.063					1.95	1.96
0.100					1.77	1.82
0.175					1.62	1.60
0.200		1.50		1.65		1.54
0.325					1.29	1.28

* Inaccurate, because of small differences in pH; see the text (p. 74).

0.152, containing 1 mM phosphate and 1.25 mM Ca not combined with protein, approximately 0.055 mM $CaHPO_4$ is present.

SUMMARY

Phosphoric acid was titrated electrometrically in the presence of KCl, NaCl, $MgCl_2$, and $CaCl_2$. No differences were observed up to the addition of 1 equivalent of NaOH. Thereafter, the

mixtures containing $MgCl_2$ or $CaCl_2$ were more acid than the corresponding mixtures containing NaCl or KCl. The results can be formulated as being due to the formation of slightly dissociated $MgHPO_4$ and $CaHPO_4$. The value for the negative logarithm of the dissociation constant of $MgHPO_4$ is approximately $2.50 - 2.15\sqrt{\mu}$. $CaHPO_4$ shows a slightly greater tendency to dissociate.

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A REVALUATION OF THE METHOD DESCRIBED BY GOODHART AND SINCLAIR FOR THE DETERMINATION OF BLOOD COCARBOXYLASE VALUES*

By ROBERT GOODHART

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Sinclair and I (1, 2) recently (1939, 1940) described a method for the determination of the cocarboxylase in blood and reported values for man, in health and disease, and for various animals. Our method was a slight modification of that described by Ochoa and Peters (3) for the estimation of cocarboxylase in boiled tissue extracts. As a result of this work, which was done in England, we concluded that the method we used furnished a reliable and rapid means for the determination of cocarboxylase in small samples (2 ml.) of oxalated blood.

While attempting to apply this method to the determination of blood cocarboxylase levels in patients at Bellevue Hospital I found that several modifications were necessary, owing largely, if not entirely, to the differences in the dried yeasts used as sources of carboxylase. The purpose of this paper is to demonstrate the nature and cause of these modifications, and the utility of the method, as modified, for the determination of blood cocarboxylase values.

Method

Nine different preparations of air-dried bakers' yeast[1] were tried before one was found which gave consistent positive anaerobic carbon dioxide evolution proportionate to the amount of

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[1] Supplied by The Fleischmann Laboratories, New York.

added cocarboxylase.   It has not been possible to obtain in New York City any sample which retained any carboxylase activity after being dried with acetone, following treatment with dibasic sodium phosphate.   This necessitated washing fresh portions of the dried yeast for each experiment.   Thus, a new "activity curve" had to be incorporated with each experiment.   In addition, because of the high vitamin B_1 content of these yeasts, it was necessary to increase the dilution with dibasic sodium phosphate used in washing to avoid enormously high "controls."   It was found that the best results were obtained by washing 0.5 gm. of dried yeast three times with 50 ml. of 0.1 M dibasic sodium phosphate and once with distilled water.   For each washing the yeast was stirred not over 2 and centrifuged not over 5 minutes. In order that the washed yeast might not stand too long, and thus lose its activity, the work was so arranged that the yeast could be added to the flasks as soon as it was washed and immediately before the flasks were attached to their manometers and placed in the bath.

The production of carbon dioxide from pyruvic acid, in the presence of the yeast preparation (as a source of carboxylase) and excess vitamin B_1, was measured in Warburg manometers.   To each 15 ml. bottle were added in order sufficient phosphate buffer (pH 6.2) to bring the total volume to 3.0 ml.; 1.0 ml. of blood containing 0.2 per cent neutral potassium oxalate; 0.1 ml. of a solution containing $MgCl_2$, $MnCl_2$, and $CaCl_2$ (0.1 mg. of Mg, 3 γ of Mn, 0.96 mg. of Ca); 0.2 ml. of vitamin solution (containing 10 γ of vitamin B_1); 1.0 ml. of yeast suspension (100 mg. of the yeast preparation in 0.1 M phosphate buffer, pH 6.2).   The $CaCl_2$ was omitted from control flasks that did not contain blood.   After equilibration in the bath at 28° in an atmosphere of nitrogen, the reaction was started by tipping in from the side bulb 0.2 ml. of sodium pyruvate (pH 6.2, containing 5 mg. of pyruvic acid).   All determinations were made in duplicate.

Results

Effect of Yeast on Sensitivity—With this experimental procedure the results obtained with added cocarboxylase and 10 γ of vitamin B_1 (in the absence of blood) were consistently in the neighborhood of those given in Table I, Column 2.   With the bakers' yeast

used in England, never less than 80 microliters of carbon dioxide were evolved, and generally more than 140 microliters, from 0.5 γ of cocarboxylase in the presence of 10.0 γ of vitamin B_1. Sinclair and I found, also, as did Ochoa and Peters (3), that the top of the curve was approached with 2.0 γ of cocarboxylase. In the experiment recorded in Table I, 0.5 γ gave only 15 microliters of carbon dioxide and the top of the curve was not reached with 8.0 γ.

As a result of tests kindly performed for me by A. Walti of Merck and Company, Inc., Rahway, New Jersey, it did not seem likely that the poor evolution of carbon dioxide obtained in these experiments could have been due in any large part to adulteration

TABLE I

Effect of Addition of Cocarboxylase and of Boiled Human Blood on Gas Production from Washed Yeast in Presence of 10 γ of Vitamin B_1

Cocarboxylase (1)	CO_2 in 30 min. (2)	CO_2 from 1.0 ml. blood in 30 min. (3)
γ	microliters	microliters
0.5	15	12.6
1.0	30.4	19.2
2.0	50.6	28.9
4.0	79.5	33.8
8.0	135.7	

of the cocarboxylase with inert material. The more reasonable conclusion was that the available yeast preparations were much less active sources of cocarboxylase than those used in England by Sinclair and myself.

Ochoa and Peters (3), working in the same laboratory as Sinclair and I, and using the same yeast,[2] found that a nearly maximum stimulating effect was exerted by 15 γ of vitamin B_1 in the type of system under discussion. In an attempt to increase the carbon dioxide evolution from cocarboxylase with the less active yeast available here, 10, 20, 30, 40, and 50 γ of vitamin B_1 were added. It was found that the carbon dioxide evolution from 0.5 γ of cocarboxylase increased with added vitamin B_1 up to 40 γ, the curve

[2] Supplied by the Distillers Company, Ltd., England.

being level between this point and 50 γ.   A carbon dioxide evolution of 70 microliters was obtained from 0.25 γ, and 131 microliters from 0.5 γ of cocarboxylase, in the presence of 50 γ of vitamin B_1.

Effect of Yeast on Specificity—It will be noted from Table I that the amounts of carbon dioxide evolved by 1 ml. portions of boiled blood samples, in the presence of 10 γ of vitamin B_1 and without added cocarboxylase, were equivalent to those obtained with from 0.42 to 1.11 γ of cocarboxylase under the same conditions.   If the gas produced by the blood actually represented cocarboxylase activity, these blood samples from hospital patients must have contained from 42 to 111 γ of cocarboxylase per 100 ml.   That,

TABLE II

Effect of Pyruvate upon Evolution of Gas from Boiled Whole Blood and Plasma,
in Presence of Washed Yeast

CO_2 from 1 ml. blood (1)	CO_2 from 1 ml. blood + 5 mg. pyruvic acid (2)	Evolved CO_2 due to pyruvic acid (3)	Cocarboxylase in 100 ml. blood (4)	CO_2 from 1 ml. plasma (5)	CO_2 from 1 ml. plasma + 5 mg. pyruvic acid (6)	Evolved CO_2 due to pyruvic acid (7)
microliters	*microliters*	*microliters*	γ	*microliters*	*microliters*	*microliters*
22.5	39.5	17.0	9.5	43.4	43.8	−0.4
28.5	33.5	5.0	3.0	37.7	38.7	+1.0
				42.1	41.8	−0.3
16.9	24.1	7.2	4.0	33.5	29.0	−4.5
22.7	30.7	8.0	4.5			
15.5	23.7	8.2	4.5			

in fact, a large part of the carbon dioxide evolved was not produced by the decarboxylation of pyruvic acid, and was no function of cocarboxylase, is demonstrated by the results presented in Table II.

In Column 1 are recorded the microliters of carbon dioxide evolved in $\frac{1}{2}$ hour by five boiled blood samples (1 ml. portions) in the presence of alkaline washed yeast, 50 γ of vitamin B_1, phosphate buffer (pH 6.2), and the mixture of ions described previously but without added pyruvic acid.   The carbon dioxide produced by the same blood samples in the presence of 5 mg. of pyruvate is noted in Column 2.   The amount of gas liberated by the decarboxylation of pyruvate, which alone can be a function of the blood cocarboxylase, consists of the difference between the figures in

Columns 1 and 2 (Column 3). Translated into γ of cocarboxylase per 100 ml. of blood (Column 4), the figures in Column 3 now give values for these blood samples compatible with the results obtained by Sinclair and myself. (It should be noted at this time that I have been able to confirm our previous finding that boiled blood shows no adjuvant action upon added cocarboxylase.)

Columns 5 and 6 show the results obtained with 1 ml. portions of the plasma of these same blood samples. The gas evolved by the plasma was consistently greater than that from the corresponding whole blood but the addition of pyruvate to the plasma in no instance caused an increase in the amount of carbon dioxide produced. Thus, although the absence of gas production by pure

TABLE III

Microliters of CO_2 Evolved by Boiled Whole Blood and Boiled Washed Erythrocytes with and without Pyruvic Acid, in Presence of Washed Yeast

1 ml. blood	1 ml. blood + pyruvic acid	Evolved CO_2 from pyruvic acid	1 ml. washed r.b.c.	1 ml. washed r.b.c. + pyruvic acid	Evolved CO_2 from pyruvic acid
(1)	(2)	(3)	(4)	(5)	(6)
10.5	41.1	30.6	0	27.0	27.0
22.7	30.7	8.0	0	8.9	8.9
21.7	30.5	8.8	2.0	17.5	15.5
35.4	47.9	12.5	0	13.3	13.3

plasma, with or without pyruvate, as reported by Sinclair and myself, is not borne out by these experiments with a different sample of bakers' yeast, the finding that there is no cocarboxylase in plasma which can be detected by this method is adequately confirmed.

That a greater amount of carbon dioxide was evolved by plasma without pyruvate than by whole blood suggested that perhaps all of the interfering substance might be in the plasma. To test this hypothesis, blood cells were washed three times with 0.8 per cent NaCl and then, after dilution with phosphate buffer (pH 6.2) to their concentration in the whole blood, were run with and without pyruvate, along with the corresponding whole blood samples. The figures in Table III show clearly that under these experimental conditions washed blood cells do not evolve carbon dioxide

without pyruvate. This experiment demonstrates also the fact that all the cocarboxylase in blood is to be found within the blood cells (compare Columns 3 and 6).

In an effort to discover the nature of the substance in plasma which produced carbon dioxide in the absence of pyruvate, with the yeast now being used, the effect of the addition of a variety of substances was tried.

Nicotinic acid[3] again proved to exert no effect in amounts up to and including 400 γ (3 cc. total fluid volume), but definite increases in the amount of carbon dioxide evolved were observed with 600 and 800 γ. Cozymase (diphosphopyridine nucleotide)[3] was without any effect in amounts up to 30 γ, with or without

TABLE IV

Effect of Added Glucose on Evolution of CO_2 from Alkaline Washed Yeast, in Presence of 5 Mg. of Pyruvic Acid and 10 γ of Vitamin B_1 and on CO_2 Produced through Action of Cocarboxylase

Glucose, mg	0	0.5	1.0	2.0	3.0
	micro-liters	micro-liters	micro-liters	micro-liters	micro-liters
CO_2 produced by washed yeast in 30 min	29.8	32.1	41.1	183.3	222.0
CO_2 produced by 0.5 γ cocarboxylase	38.5		37.7		
" " " 0.5 " "	25.5			24.2	

added hexose diphosphate.[3] Riboflavin[3] was tried in amounts from 50 to 150 γ, and no change in the evolution of carbon dioxide was noted. Ammonium sulfate definitely depressed the carbon dioxide production in amounts of 5, 10, and 15 mg., the degree of depression being directly proportional to the concentration of the salt.

In the presence of 100 γ of magnesium, any increase in the manganese present over 3 γ, up to 20 γ, was without effect.

Sinclair and I found that no increase in gas production could be observed in the presence of added glucose up to 1 mg. With the yeast I was now using I noted a slight but definite increase with as little as 0.5 mg. of glucose, and an enormous increase with 2 mg. or more. This increase was absolute and independent of added

[3] Supplied by Merck and Company, Inc., Rahway, New Jersey.

cocarboxylase (Table IV), in this respect resembling the substance in boiled blood which was causing gas production independently of added cocarboxylase and pyruvic acid. The resemblance included also a common depression by iodoacetate (5 mg.) and sodium fluoride (15 mg.) and an immunity to sodium cyanide (15 mg.), phenomena true also of cocarboxylase.

From these facts, plus the observation that the "non-specific" gas production from blood tended to decrease if the blood were permitted to stand an hour or more at room temperature before boiling, the conclusion was drawn that the gas evolved by heated blood with alkaline washed yeast, in the absence of pyruvate, was due to glycolysis. The yeast used in these experiments apparently retained a much greater glycolytic activity after being washed with alkaline phosphate than that used previously by Sinclair and myself.

As a result of these experiments, the procedure for the determination of blood cocarboxylase values was modified as follows: (1) 50 γ of vitamin B_1 are used instead of 10 γ. (2) The determinations are made on blood cells which have been washed three times with equal volumes of 0.8 per cent sodium chloride and then diluted with phosphate buffer (pH 6.2) to their concentration in the whole blood, instead of on whole blood. The calcium chloride is omitted when washed blood cells are used.

Normal Blood Cocarboxylase Values

The blood cocarboxylase was determined, by the modified procedure, in twenty-eight boys, all patients on the children's wards of the Psychiatric Division of Bellevue Hospital. Clinically they were all apparently normal with respect to obvious vitamin deficiency states. Their ages ranged from 5 to 15 years. Their cocarboxylase levels, in terms of the synthetic cocarboxylase used as a standard, varied from 4.5 to 10 γ per 100 ml. of blood, with a mean of 7.0 γ ± 1.53 s.d. Sinclair and I assumed a normal value of 7.0 γ ± 2.1 s.d. as a result of our findings in thirty-six estimations on twenty-six healthy adults.

SUMMARY

1. It has been found advisable to modify the method for determining blood cocarboxylase, described by Goodhart and Sinclair,

by making the determinations on washed blood cells, instead of whole blood, and by running these estimations in the presence of 50 γ of vitamin B_1 instead of 10 γ.

2. The presence of all the blood cocarboxylase within the blood cells has been confirmed.

3. Blood cocarboxylase levels were determined in twenty-eight "normal" boys and the mean was found to be 7.0 γ ±1.53 s.D.

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THE EFFECT OF SUPPLEMENTARY CYSTINE AND METHIONINE ON THE PRODUCTION OF FATTY LIVERS BY RATS ON HIGH FAT DIETS CONTAINING CASEIN OR EDESTIN

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It is generally accepted that methionine exerts a lipotropic effect on the liver lipids of rats (1–3) or mice (4) on low protein-high fat diets. In the first of a series of papers from this laboratory (1) Tucker and Eckstein suggested that the lower lipid values of rats obtained by Channon and Wilkinson (5) when the dietary protein was increased from 5 to 20 per cent might be ascribed to the methionine content of the protein (casein). In a later paper Tucker and Eckstein (3) suggested that amino acids other than those containing sulfur might exert some influence on the production of livers with a high fat content, since rats on a diet containing 5 per cent gliadin and 40 per cent lard supplemented with 0.5 per cent cystine had practically the same amounts of liver lipids as rats on an unsupplemented 5 per cent gliadin diet. This was unexpected, since Beeston and Channon (6) had previously reported that the fat content of livers was markedly increased when a high fat diet containing 5 per cent casein was supplemented with as little as 0.2 per cent cystine. Quite recently Best and Ridout (7) supplemented a high fat diet containing protein in the form of 5 per cent dried beef muscle with varying amounts of *dl*-methionine and observed that the liver fat of the rats was decreased practically to the same extent when

0.125 per cent of the *dl*-methionine was added as when 1 per cent of the racemic mixture was included. They also studied the lipotropic action of the *d* and *l* forms of this acid and reported that the addition of as little as 0.15 per cent of either one of the isomers to the basal diet resulted in a lowering of the lipid content of the livers. These findings are not in accordance with those of Channon *et al.* (2) who had previously reported that no appreciable lipotropic effect was demonstrable unless the supplementary level of the methionine of a low protein diet (casein) was raised to 0.5 per cent. Best and Ridout (7) also reported that, whereas fatty livers were not obtained when their basal diet was supplemented with 30 per cent casein, other rats on this same basal diet to which amounts of cystine and methionine equal to those in the 30 per cent casein supplement had been added developed fatty livers. They concluded that "factors other than cystine and methionine are involved in the explanation of the lipotropic action of protein." The amounts of cystine and methionine added by Best and Ridout to their basal diet were 0.1 and 0.96 per cent respectively. As has already been mentioned, these same authors reported that a drop in liver lipids occurred when as little as 0.125 per cent of methionine was added to this same basal diet. The inclusion of as little as 0.1 per cent of cystine apparently inhibited the lipotropic effect of the supplementary methionine (0.96 per cent).

During the past year we have accumulated additional information related to the effects of supplementary cystine and methionine on liver lipids. Some of the data obtained are at variance with some of those presented by Best and Ridout and it therefore seems desirable to report on these differences.

As usual young white rats (100 to 120 gm.) of a commercial strain were employed as experimental animals. The conduct of the experiments and analytical procedure, which were essentially the same as those employed by the other workers referred to herein, have already been described elsewhere (1). The duration of the experiments was approximately 3 weeks. The diets employed are described in Table I and the data obtained summarized in Table II. The cystine was prepared from hair and the methionine obtained from the Department of Chemistry of the University of Illinois.

It is clear from Tables I and II that Diets 2 and 3 which contain the same amounts of cystine and methionine[1] exhibited similar lipotropic actions, the average liver lipid contents being,

TABLE I

Experimental Diets

All diets contained 2 per cent agar, 40 per cent lard, and 5 per cent salt mixture* in addition to the ingredients listed in the table. Each animal received 1 dried yeast tablet (400 mg.) and 1 drop of cod liver oil daily.

Diet No.	Casein	Edestin	Glucose	l-Cystine added	dl-Methionine added	Cystine in 100 gm. diet	Methionine in 100 gm. diet
	per cent	per cent	per cent	per cent	per cent	mg.	mg.
1	5		48			17	155
2	20		33			68	620
3	5		47.5	0.051	0.465	68	620
4	15		38			51	465
5	5		47.66	0.034	0.310	51	465
6		5	48			60	117.5
7		5		0.008	0.5035	68	620

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 37, 572 (1919).

TABLE II

Influence of Supplementary Casein, Methionine, and Cystine on Liver Lipid Content of Rats on Low Protein-High Fat Diets

Diet No.	Cystine in 100 gm. diet	Methionine in 100 gm. diet	No. of rats	Liver lipids*
	mg.	mg.		per cent
1	17	155	21	23.7 (9.4-43.9)
2	68	620	23	14.4 (6.8-27.6)
3	68	620	10	11.5 (7.1-15.5)
4	51	465	11	25.9 (18.5-33.7)
5	51	465	12	18.1 (11.3-25.0)
6	60	117	12	31.8 (14.4-46.1)
7	68	620	12	12.5 (7.1-18.7)

* The values in parentheses are the variations for the individual rats.

14.4 and 11.5 per cent respectively. This similarity in effect was obtained in spite of the marked difference in the protein and

[1] The amounts of cystine and methionine in the diets containing casein and edestin were calculated from the analysis given by Kassell and Brand (8).

consequently the non-sulfur-containing amino acid content of the two diets, Diet 2 containing 4 times the amount of these present in Diet 3.   Table II likewise shows that increasing the level of casein from 5 to 15 per cent did not influence the liver lipid content, since the average values 23.7 and 25.9 per cent are in close agreement with each other.   When Diet 5 which contained 5 per cent casein and the same amounts of cystine and methionine as the 15 per cent casein diet was fed, an average value of 18.1 per cent liver lipids was obtained.   This figure is lower than the one recorded for the rats on the 15 per cent casein diet, even though the content of the non-sulfur-containing amino acids of the latter was 3-fold that of Diet 5.

Edestin, which contains more cystine and less methionine than casein, gave the highest average value (31.8 per cent) recorded in Table II.   This was anticipated, because the former amino acid is known to cause an increase in the fat content of the liver, whereas the latter has an opposing effect.   However, when Diet 6 (5 per cent edestin) was supplemented with amounts of cystine and methionine equivalent to those present in a 20 per cent casein diet, a lipotropic action (12.5 per cent liver lipids) was demonstrated.

In experiments with mice it had been previously demonstrated in this laboratory (4) that fatty livers were obtained on high fat diets containing as much as 20 per cent of arachin.   In fact little if any difference was observed between the liver lipid contents of mice on a 5 per cent arachin diet and those fed the protein at a 20 per cent level.   In contrast to these observations a 20 per cent casein diet exerted a lipotropic action on the livers of the mice whereas one containing only 5 per cent casein did not. The difference between the casein and arachin effects was ascribed to the low methionine content (0.77 per cent) of the latter (9). When a 5 per cent arachin diet was supplemented with methionine to the extent that the total methionine content of the supplemented diet was equal to that of the 20 per cent casein diet, an unquestionable lipotropic action was secured.   Our experience with rats on gliadin diets was practically the same as that with casein, arachin, and edestin.   Fatty livers were almost invariably obtained when this protein was fed at a 5 per cent level, but a distinct lipotropic effect was obtained when the basal 5 per cent

gliadin diet was so supplemented that its methionine content was equivalent to that of a 20 per cent casein diet.

It is of interest to note that the supplemented gliadin diet mentioned above contained 137 mg. per cent cystine and 682 mg. per cent methionine. Practically normal livers (7.1 per cent total lipids) were obtained. In contrast to this Best and Ridout (7) reported that fatty livers were obtained when a basal high fat diet containing protein in the form of 5 per cent dried beef muscle was supplemented with 100 mg. of cystine and 960 mg. of methionine; i.e., smaller amounts of cystine and larger amounts of methionine than were present in our supplemented diet. Unfortunately Best and Ridout do not give analytical figures for the dried beef powder. In the absence of such data it is futile to attempt to explain the difference between their data and ours.

The data presented in Table II confirm those previously published from this laboratory, since a lipotropic action was invariably obtained when diets low in protein and high in fat were supplemented with methionine and cystine to the extent that the sulfur amino acid content of the supplemented diets was equal to that of a diet containing 20 per cent casein. The latter diet of course contains much larger amounts of the non-sulfur-containing amino acids than the supplemented diets but under the conditions obtaining in our experiments, i.e. when the cystine and methionine contents of the diets are 68 and 620 mg. per cent respectively, the inclusion of the larger amounts of the non-sulfur-containing amino acids did not increase the lipotropic effect of the diet.

SUMMARY

The lipotropic effect in rats of a low protein (5 per cent casein or edestin), high fat (40 per cent lard) diet supplemented with methionine and cystine to the extent that the sulfur amino acid content was equivalent to that of a 20 per cent casein diet was similar to that observed when the level of the casein in the unsupplemented diet was raised to 20 per cent.

Fatty livers were obtained when the casein content of the unsupplemented high fat diet was decreased to 15 or 5 per cent. The total lipid content of the livers of rats on the unsupplemented 15 per cent casein diet was slightly larger than that of rats on a 5 per cent casein diet to which such amounts of the sulfur-containing amino acids had been added that the methionine content of the supplemented diet was equivalent to that in the 15 per cent casein diet.

The effects obtained on the lipid content of the rats on the different diets employed are explainable on the basis of the methionine and cystine contents of the diets.

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THE REACTION OF THE ESTERS OF PHENYLAMINO-ACETIC ACID AND OF PHENYLALANINE ON THE RANEY CATALYST

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In previous communications[1] the results of the reduction of the esters of aliphatic α-amino acids first over the copper chromite catalyst of Adkins and later over Raney's nickel catalyst were reported. The latter results encouraged experiments on the application of the Raney nickel catalyst for the reduction of phenylated α-amino acids for whose reduction the copper chromite catalyst is ineffective.

The present communication deals with the action of Raney's catalyst under a pressure of 150 atmospheres and under varying temperatures on the esters of aminophenylacetic acid and of phenylalanine.

In the main features the behavior of these esters paralleled that of leucine ester, although it was not identical. The phenylated ester contains two groups subject to the attack of hydrogen under the influence of catalysts and it was envisaged that conditions might be found under which one group would be affected preferentially. This expectation actually was realized. At a temperature of 40° the preferential reaction is the reduction of the —COOCH$_3$ group to a CH$_2$OH group but it is possible to accomplish in addition to the reduction of the COOR group also a complete hydrogenation of the phenyl group. All that is required for this end is to prolong the operation from 6 to 24 or on occasion even to 36 hours, depending on the quantity of the catalyst employed.

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At higher temperatures the interval in which one reaction predominates was not sought and hence no answer can be given to the question as to the relative velocities of the two reactions. Under the conditions of the present investigation at the temperatures of 135° and 185° only cyclohexyl derivatives were obtained.

At the lower of the two temperatures, only one product was obtained; namely, N-dimethyl-2-aminocyclohexylethanol and 2-amino-2-hexahydrobenzylethanol.

At the higher temperature, N-dimethylmethylcyclohexylcarbinamine and N-dimethylmethylhexahydrobenzylcarbinamine respectively and substituted N,N'-dimethylpiperazines were obtained in pure form. The presence of the aminoethanol derivatives was made probable but they were not isolated in pure form.

The mechanism of piperazine formation is probably similar to that in the series of aliphatic esters, for in the absence of catalyst, the esters of the phenylated α-amino acids in methanol solution condense into ketopiperazines.

It is significant that at 40° the ester of the active aminophenylacetic acid led to an active 2-amino-2-phenylethanol or to an active 2-amino-2-cyclohexylethanol. The reduction of the ester of phenylalanine at this temperature has not yet been tested.

SUMMARY

1. The nature of the products formed on reduction of the esters of aminophenylacetic acid and of phenylalanine over Raney's nickel catalyst depends on the temperature and on the duration of the reaction.

2. At 40° the ester of aminophenylacetic acid is reduced either to 2-amino-2-phenylethanol or 2-amino-2-cyclohexylethanol, depending on the duration of the reaction. The optically active ester leads to optically active aminophenylethanol. At higher temperature, N-dimethylcarbinamines and substituted N,N'-dimethylpiperazines are the principal products of the reaction.

3. It is suggested that over Raney's nickel catalyst the mechanism of piperazine formation from the phenylated esters of α-amino acids is similar to that from the esters of aliphatic α-amino acids.

4. In methanol solution in the absence of catalyst the esters of

phenylated α-amino acids heated to 160–170° are condensed into ketopiperazines.

Action of Raney's Catalyst on d-α-Aminophenylacetic Acid Ethyl Ester in Presence of Hydrogen at 40°—20 gm. of α-amino-phenylacetic acid, $[\alpha]_D^{25} = -125°$ (in 20 per cent hydrochloric acid), were converted into its ethyl ester in the usual manner. B.p., 95–98°; $p = 1$ mm.; $n_D^{25} = 1.5149$; yield, 6 gm.; $d_4^{25} = 1.08$.

The substance had the following composition.

4.201 mg. substance: 10.340 mg. CO_2 and 2.730 mg. H_2O
7.390 "   "   : 0.514 cc. N_2 at 27°, $p = 750$ mm.

$C_{10}H_{13}O_2N$.   Calculated.   C 67.00, H 7.25, N 7.81
179.1   Found.   " 67.12, " 7.27, " 7.81

$$[\alpha]_D^{25} = \frac{-123°}{1 \times 1.08} = -113° \text{ (homogeneous)}$$

Reduction to Aminophenylethanol—31 gm. of Raney's catalyst (18 cc. of centrifuged catalyst) were added to a solution of 5.5 gm. of α-aminophenylacetic acid ethyl ester, $[\alpha]_D^{25} = -113°$ (homogeneous) in 125 cc. of absolute methanol. This was reduced with hydrogen at a pressure of 2200 pounds per sq. inch and at 40° for 9 hours. The product distilled at 91–98°; $p = 0.1$ mm.; yield, 2.5 gm.; $n_D^{25} = 1.5567$.

$$[\alpha]_D^{25} = \frac{-1.36° \times 100}{1 \times 9.06} = -15.0° \text{ (in methanol)}$$

In another experiment, an ester of $[\alpha]_D^{25} = -52.4°$ (homogeneous) gave an aminophenylethanol of

$$[\alpha]_D^{25} = \frac{-0.59° \times 100}{1 \times 7.54} = -7.8° \text{ (in methanol)}$$

$n_D^{25} = 1.5558$.

In the original experiment an ester of $[\alpha]_D^{25} = -65.3°$ (homogeneous) gave an aminophenylethanol of

$$[\alpha]_D^{25} = \frac{-0.41° \times 100}{1 \times 7.3} = -5.61° \text{ (in methanol)}$$

4.650 mg. substance: 912 mg. CO_2 and 3.440 mg. H_2O
C$_8$H$_{11}$ON.   Calculated.   C 70.02, H 8.09
137.1   Found.   " 69.86, " 8.27

Picrate of 2-Amino-2-Phenylethanol—2-Amino-2-phenylethanol, $[\alpha]_D^{25} = -7.8°$ (in methanol), was converted into the picrate as usual. M.p., 208–210°.

$$[\alpha]_D^{25} = \frac{-0.11° \times 100}{1 \times 6} = -1.8° \text{ (in methanol)}$$

2 drops of concentrated hydrochloric acid were added to the solution and the rotation of the hydrochloride then was

$$[\alpha]_D^{25} = \frac{-0.24° \times 100}{1 \times 2.3} = -10°$$

5.048 mg. substance: 8.515 mg. CO_2 and 1.795 mg. H_2O
4.565   "   "   : 0.630 cc. N_2 at 29° and 752 mm.
$C_{14}H_{14}O_8N_4$. Calculated. C 45.88, H 3.85, N 15.35
366.1   Found.   " 45.96, " 3.78, " 15.42

Reduction to Aminocyclohexylethanol—2 gm. of *dl*-α-aminophenylacetic acid ethyl ester were dissolved in 100 cc. of methanol and 12 cc. (17.5 gm.) of centrifuged Raney's catalyst were added. This was reduced at a pressure of 2200 pounds for 18 hours at 40°. The substance distilled from a bath temperature of 130–135°; $p = 0.5$ mm. Yield, 1 gm. of crystalline material; $n_D^{25} = 1.5010$ (melted crystals).

4.299 mg. substance: 10.610 mg. CO_2 and 4.525 mg. H_2O
$C_8H_{17}ON$. Calculated. C 67.07, H 11.96
143.1   Found.   " 67.30, " 11.77

1 gm. of ester, $[\alpha]_D^{25} = -113°$ (homogeneous), was dissolved in 100 cc. of methanol and 9.5 cc. of Raney's catalyst (17 gm.) were added. This was reduced at a pressure of 2200 pounds for 44 hours at 40°. The substance distilled from a bath temperature of 95–105°, $p = 0.1$ mm., and crystallized.

$$[\alpha]_D^{25} = \frac{-0.60° \times 100}{1 \times 12.6} = -4.8° \text{ (in methanol)}$$

4.715 mg. substance: 11.705 mg. CO_2 and 4.885 mg. H_2O
Found. C 67.69, H 11.59

Action of Raney's Catalyst on dl-Aminophenylacetic Acid Ethyl Ester in Presence of Hydrogen at 135°—7 gm. of Raney's catalyst were added to a solution of 5 gm. of *dl*-aminophenylacetic acid ethyl ester in 120 cc. of absolute methanol. The reduction was

carried out at an initial hydrogen pressure of 2200 pounds per sq. inch at 135° for 9 hours.

Further treatment was as described in the previous communication.[1]

The final product distilled at 140°; $p = 20$ mm. The yield was 4 gm.; $n_D^{25} = 1.4852$.

The substance had a composition agreeing with that calculated for *N-dimethyl-2-amino-2-cyclohexyl-1-ethanol*.

5.270 mg. substance: 13.554 mg. CO_2 and 5.720 mg. H_2O
4.381 "   "   : 0.514 cc. N_2 at 27°, $p = 754$ mm.
          $C_{10}H_{21}ON$.   Calculated.   C 70.11, H 12.36, N 8.18
          171   Found.   " 70.13, " 12.14, " 8.32

Preparation of Picrate of N-Dimethyl-2-Amino-2-Cyclohexyl-1-Ethanol—0.2 gm. of distilled N-dimethyl-2-aminocyclohexyl-1-ethanol was dissolved in 1 cc. of ether and an ether solution of 0.2 mg. of dry picric acid was added.

After the material had stood for several hours, the picrate had completely crystallized. A constant melting point of 92–93° was reached after the recrystallization from ethanol.

The substance had a composition agreeing with that calculated for the *picrate of N-dimethyl-2-amino-2-cyclohexylethanol*.

5.548 mg. substance: 9.770 mg. CO_2 and 3.005 mg. H_2O
3.990 "   "   : 0.492 cc. N_2 at 27°, $p = 759$ mm.
          $C_{16}H_{24}O_8N_4$.   Calculated.   C 47.97, H 6.04, N 14.00
          400   Found.   " 48.02, " 6.05, " 14.01

Action of Raney's Catalyst on dl-Aminophenylacetic Acid Ester at 185°—8 gm. of Raney's catalyst were added to a solution of 4 gm. of *dl*-aminophenylacetic acid methyl ester in 40 cc. of methanol. This was reduced with hydrogen at an initial pressure of 2200 pounds per sq. inch and a temperature of 185° for 9 hours.

Further treatment was similar to that described in the previous sections.

Fraction I—B.p., 80° at atmospheric pressure; yield, 0.7 gm. The substance had a composition agreeing with that calculated for *N-dimethyl-1-amino-1-cyclohexylethane* (methylcyclohexylcarbinamine).

2.212 mg. substance: 8.225 mg. CO_2 and 3.502 mg. H_2O
5.700 "   "   : 0.446 cc. N_2 at 29°, $p = 764$ mm.
          $C_{10}H_{21}N$.   Calculated.   C 77.41, H 13.54, N 9.03
          155.2   Found.   " 77.68, " 13.45, " 8.37

Picrate of N-Dimethyl-1-Amino-1-Cyclohexylethane—0.2 gm. of distilled N-dimethylaminocyclohexylethane was converted into the picrate. On recrystallization from methanol-ether, its melting point was 131° and its composition was as follows:

5.525 mg. substance: 10.090 mg. CO_2 and 3.095 mg. H_2O
4.190 "   "   : 0.539 cc. N_2 at 27°, $p = 761$ mm.
$\qquad$ $C_{16}H_{24}O_7N_4$. Calculated. C 49.98, H 6.29, N 14.57
$\qquad$ 384.2   Found.   " 49.80, " 6.27, " 14.65

Fraction II—The mother liquor was distilled under reduced pressure. The fraction boiling at 140° and 5 mm. consisted of a mixture of intermediate products. On a few occasions on refractionation it was possible to obtain substances approaching in composition that of *N-dimethyl-2-amino-2-cyclohexylethanol.* A pure product was not obtained.

Fraction III—B.p., 150°; $p = 5$ mm.; yield, 1.9 gm. The substance had a composition agreeing with that calculated for *N,N'-dimethyl-2,5-dicyclohexylpiperazine.*

4.808 mg. substance: 13.690 mg. CO_2 and 5.405 mg. H_2O
4.250 "   "   : 0.367 cc. N_2 at 27.5°, $p = 761$ mm.
$\qquad$ $C_{18}H_{34}N_2$. Calculated. C 77.69, H 12.23, N 10.07
$\qquad$ 278.3   Found.   " 77.64, " 12.57, " 9.73

Picrate of N,N'-Dimethyl-2,5-Dicyclohexylpiperazine—0.2 gm. of distilled N,N'-dimethyldicyclohexylpiperazine was converted into the picrate. The substance was recrystallized from an ether-ethanol solution until a constant melting point of 230–235° was obtained. The substance had a composition agreeing with that calculated for the picrate of *N,N'-dimethyl-2,5-dicyclohexyl-piperazine.*

5.214 mg. substance: 9.338 mg. CO_2 and 2.590 mg. H_2O
4.717 "   "   : 0.636 cc. N_2 at 27°, $p = 759$ mm.
$\qquad$ $C_{30}H_{40}O_{14}N_6$. Calculated. C 48.89, H 5.43, N 15.21
$\qquad$ 736.3   Found.   " 48.83, " 5.55, " 15.31

Attempted Reduction of α-Aminophenylacetic Acid Ester with Copper Chromite Catalyst—1 gm. of α-aminophenylacetic acid ester

$$[\alpha]_D^{25} = \frac{-56.6°}{1 \times 1.08} = -52.4° \text{ (homogeneous)}$$

was dissolved in 50 cc. of methanol and 1 gm. of Adkins' copper chromite catalyst was added. This was shaken with hydrogen at a pressure of 2200 pounds for 9 hours at 100°. The product, after removal of the methanol, was distilled at a pressure of 0.6 mm. A fraction boiling below 100° consisted of benzaldehyde. When the temperature was raised, the remainder decomposed.

Action of Raney's Catalyst on dl-Phenylalanine Methyl Ester in Methanol in Presence of Hydrogen at 185°—6 gm. of Raney's catalyst were added to a solution of 4 gm. of *dl*-phenylalanine methyl ester in 50 cc. of absolute methanol and shaken in the presence of hydrogen under the same conditions as in the case of phenylaminoacetic acid.

Fraction I—B.p., 90°; $p = 10$ mm.; yield, 0.8 gm. The substance had a composition agreeing with that calculated for *N-dimethyl-1-amino-1-hexahydrobenzylethane.*

4.403 mg. substance: 12.600 mg. CO_2 and 5.415 mg. H_2O
6.722 "   "   : 0.519 cc. N_2 at 28°, $p = 762$ mm.
          $C_{11}H_{23}N$. Calculated. C 78.01, H 13.71, N 8.28
          169.2   Found.   " 78.03, " 13.76, " 8.27

Picrate of N-Dimethyl-1-Amino-1-Hexahydrobenzylethane—0.2 gm. of distilled N-dimethyl-1-amino-1-hexahydrobenzylethane was converted into the picrate. On recrystallization from an ether-ethanol solution a melting point of 145–146° was obtained.

5.624 mg. substance: 10.495 mg. CO_2 and 3.190 mg. H_2O
4.800 "   "   : 0.598 cc. N_2 at 27°, $p = 761$ mm.
          $C_{17}H_{26}O_7N_4$. Calculated. C 51.23, H 6.56, N 14.06
          398.2   Found.   " 50.88, " 6.34, " 14.18

Fraction II had a composition similar to that of the corresponding fraction of the phenylaminoacetic acid experiment.

Fraction III, b.p., 150°; $p = 5$ mm.; yield, 1.8 gm.

The substance had a composition agreeing with that calculated for *N,N'-dimethyl-2,5-dihexahydrobenzylpiperazine.*

3.614 mg. substance: 10.405 mg. CO_2 and 4.010 mg. H_2O
6.700 "   "   : 0.549 cc. N_2 at 27°, $p = 759$ mm.
          $C_{20}H_{38}N_2$. Calculated. C 78.43, H 12.41, N 9.14
          306.3   Found.   " 78.51, " 12.65, " 9.24

Picrate of N,N'-Dimethyl-2,5-Dihexahydrobenzylpiperazine—0.2 gm. of distilled N,N'-dimethyl-2,5-dihexahydrobenzylpip-

erazine was converted into the picrate. On recrystallization from ether-ethanol solution, a substance with a constant melting point of 144–146° was obtained.

> 5.396 mg. substance: 9.900 mg. CO_2 and 2.910 mg. H_2O
> 4.820 "   "   : 0.613 cc. N_2 at 27°, $p = 759$ mm.
> $C_{32}H_{44}O_{14}N_8$. Calculated. C 50.23, H 5.76, N 14.65
>   764.4   Found.   " 50.03, " 6.03, " 14.52

Condensation of Phenylaminoacetic Acid Methyl Ester into 2,5-Diphenyl-3,6-Diketopiperazine—4 gm. of *dl*-phenylaminoacetic acid methyl ester were added to 5 cc. of absolute methanol. This mixture was sealed in a bomb tube which was then heated at 160° for 9 hours.

The reaction product, which was partially crystalline, was concentrated under diminished pressure to a white crystalline mass. Yield, 2.8 gm.

After recrystallization from alcohol, the crystalline product melted at 270° and had a composition agreeing with that calculated for *2,5-diphenyl-3,6-diketopiperazine.*

> 4.192 mg. substance: 11.090 mg. CO_2 and 2.025 mg. H_2O
> 6.505 "   "   : 0.600 cc. N_2 at 24°, $p = 749$ mm.
> $C_{16}H_{14}O_2N_2$. Calculated. C 72.15, H 5.26, N 10.55
>   266.1   Found.   " 72.12, " 5.40, " 10.50

Condensation of dl-Phenylalanine Methyl Ester into Phenylalanine Anhydride—3 gm. of *dl*-phenylalanine ethyl ester were added to 4 cc. of absolute methanol. This mixture was sealed into a bomb tube which was then heated at 170° for 9 hours.

The reaction product, which was partially crystalline, was concentrated under diminished pressure to a white crystalline mass. Yield, 2.2 gm.

After recrystallization from absolute ethanol the crystalline product melted at 295–296° and had a composition agreeing with that calculated for *dl-phenylalanine anhydride.*

> 5.204 mg. substance: 13.990 mg. CO_2 and 2.915 mg. H_2O
> 6.420 "   "   : 0.535 cc. N_2 at 28°, $p = 763$ mm.
> $C_{18}H_{18}O_2N_2$. Calculated. C 73.46, H 6.12, N 9.52
>   294.1   Found.   " 73.31, " 6.26, " 9.48

THE BIOLOGICAL DEMETHYLATION OF SARCOSINE TO GLYCINE*

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While N-methylamino acids are not constituents of animal tissues, some of them can be utilized by animals. A number of indispensable amino acids (histidine (1), tryptophane (2), methionine (3), phenylalanine (4), and cystine (5)) can be replaced in the diet of growing rats by their N-methyl derivatives. Others (lysine (6), leucine (4)) are reported not to be able to support growth. Sarcosine has recently been shown by Abbott and Lewis (7) to have an effect on hippuric acid formation similar to that of glycine. It appears from these findings that these N-methylamino acids are converted by the animal into the free amino acids. It has frequently been suggested that the demethylation reaction is not a direct one, but involves oxidative deamination to the keto acid and subsequent reamination. According to this conception the methylated compounds should be treated by the animal in a way similar to that of amino acids of unnatural steric configuration. This reaction has recently been studied with the aid of two independent isotopes, deuterium and N^{15}. During the inversion of $d(-)$-phenylaminobutyric acid (8) and of $d(+)$-leucine (9) the original nitrogen had been replaced almost completely by nitrogen from other sources. If the demethylation reaction is similar to that of biological inversion, *i.e.* involves primary deamination, the isotopic nitrogen of an administered methylamino acid should be removed from the carbon skeleton. From the results of the known experiments it cannot be decided whether a replacement of the original nitrogen had occurred.

Our present findings show that during biological conversion of sarcosine to glycine the nitrogen originally attached to the carbon chain is not replaced. The experiments were carried out in the course of a study on the biological precursors of creatine.[1] Isotopic sarcosine was added to the ordinary stock diet of rats kept under conditions analogous to those when other isotopic nitrogenous compounds (dl-tyrosine (11), l(−)-leucine (12), d(+)-leucine (9), glycine (13), ammonia (14)) were administered. The animals were killed at the end of the experimental period and a

TABLE I

N^{15} Concentration in Excreta and Tissue Constituents from Rats Given Isotopic Sarcosine or Glycine

The values are calculated for an N^{15} concentration of 100 atom per cent in the glycine administered and in the sarcosine.

		Glycine fed 3 days	Sarcosine	
			Fed 3 days	Fed 6 days
Intestinal tract protein	Glycine	4.22	5.51	
	Amide N		1.76	
Liver protein	Arginine	0.78	0.89	
	Glutamic acid	0.89	1.20	1.71
	Aspartic "	0.73	1.00	
	Amide N	1.44	1.58	3.62
	Glycine	8.86		14.78
	Total N	1.40		2.80
Total urinary N		2.84	2.89	
Urinary ammonia		3.06	5.04	
" urea		2.73	3.04	

number of nitrogenous constituents were isolated and their isotope content determined. The results obtained with sarcosine were identical with those found after glycine was fed (Table I). As reported before (13), the feeding of isotopic glycine results in the presence of large amounts of isotopic glycine in the proteins of the animals, while other amino acids contain much lower concentrations of isotope. This is due to a direct replacement of glycine in the proteins by dietary glycine. The same took place after the

[1] The results of this study will be published later. A short report has appeared recently (10).

feeding of sarcosine. The glycine isolated from the proteins of liver and intestinal wall had an isotope content much higher than any other constituent.

The finding excludes the occurrence of intermediate deamination of sarcosine as a major pathway during the conversion. Such deamination has been observed with the methyl derivative of the "unnatural" $d(-)$-alanine *in vitro* (15). When dl-N-methylalanine was treated with d-amino acid oxidase under aerobic conditions, almost half was degraded to pyruvic acid and methylamine. Whether this type of reaction would occur *in vivo* with methylated "natural" amino acids is uncertain. If oxidative deamination of sarcosine had taken place in our animals, *i.e.* if methylamine were split off, the marked nitrogen would have been detached and the glycine of the proteins should have contained no marked nitrogen, or only traces. Its concentration should have been lower than that of the metabolically very active dicarboxylic acids, as was the case with the leucine of the proteins after the administration of isotopic unnatural leucine ($d(+)$-leucine). The results, considered together, can scarcely be explained in any other way than by a direct demethylation of sarcosine to form glycine.

The direct demethylation of sarcosine was so rapid that the results of the 3 day experiment were almost the same whether the isotope was given in sarcosine or in glycine; the process must have occurred during or immediately after absorption. However, we cannot exclude the possibility that a small portion of the sarcosine was directly oxidized. The experiments furthermore do not indicate whether the reaction found with sarcosine is typical also for other methylated amino acids.

EXPERIMENTAL

The isotopic sarcosine employed in this experiment was prepared from isotopic glycine as described before (16). The substance contained 4.50 atom per cent N^{15} excess.

Three male rats of 300 gm. weight were kept on our stock diet containing 15 per cent casein (12). In addition to this diet, two of the animals received 146 mg. of sarcosine, corresponding to 23 mg. of nitrogen each, per day for 3 days, while the other animal received the same addition of isotopic sarcosine for 6 consecutive days. The weight of the animals remained constant throughout

the experimental period. The procedure was therefore identical with that used in the glycine experiment (13). The methods employed for analysis and isolation of the various nitrogenous compounds were the same as those described in previous papers (12, 13). To facilitate comparison, Table I includes the corresponding values obtained in the glycine experiment. Glycine from the proteins was isolated with potassium trioxalatochromiate according to Bergmann and Niemann (17) and converted into toluenesulfonylglycine.

SUMMARY

Adult male rats of constant weight were kept on a casein-containing stock diet to which an amount of sarcosine corresponding to 23 mg. of nitrogen per day was added. The nitrogen of the sarcosine contained 4.50 atom per cent N^{15} excess. The experiments were carried out for 3 and 6 days. At the end of the feeding period the animals were killed and a number of amino acids were isolated from the proteins and their isotope content determined. The results were compared with analogous experiments in which isotopic glycine instead of sarcosine was added.

The results of the two experiments (sarcosine and glycine) were almost identical. The isotope concentration in the glycine from the proteins was high, while that of the other amino acids was much lower.

The results indicate that sarcosine is directly demethylated to form glycine without deamination. The demethylation process is very rapid and must have occurred during or immediately after absorption.

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A MODIFICATION OF THE MILLER-MUNTZ METHOD FOR COLORIMETRIC DETERMINATION OF LACTIC ACID

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The method described by Miller and Muntz (1938) has the disadvantage that the color reagent, p-hydroxydiphenyl, is added in dry form, because the authors were unable to find a suitable solvent for the substance. Such a procedure involves the weighing of an 8 mg. sample to 0.2 mg. for each determination and quantitative transfer of this small amount of material from the weighing bottle to the reaction vessel. The latter operation is made especially difficult by the physical nature of the solid.

This report describes a means of adding the reagent in solution form, with a slight necessary modification of the method. Since p-hydroxydiphenyl is weakly acidic, it will dissolve in a solution of NaOH. The addition to the reaction mixture of the small amount of sodium hydroxide necessary is sufficient to neutralize only a negligible portion of the concentrated sulfuric acid used for oxidation of the lactic acid to acetaldehyde. The intensity of color developed is slightly decreased by this procedure, but is relatively independent of the amount of reagent used. Varying the volume added from 0.010 up to 0.025 cc. produced no detectable difference in any quality of the final color.

Reagents—

1. Sulfuric acid, sp. gr. 1.84 (Baker's c.p.). Samples of this usually proved quite satisfactory, but new lots should be tested by making several determinations on standard lactate solutions and comparing the intensity of color with that produced when Kahlbaum's *pro analysi* H_2SO_4 is used. (Occasional samples have been found which produce a color considerably less intense than that given by the Kahlbaum grade). Exposure to air should be kept at a minimum.

2. *p*-Hydroxydiphenyl (Eastman).   Purify according to Miller and Muntz (1938).   Dissolve 225 mg. of the purified crystalline reagent in 10 cc. of 0.18 M NaOH.   This is almost a saturated solution; if crystals form on standing, they should be redissolved by warming the solution before use.   A slight pink or yellow color may develop on standing but is not significant; solutions have been kept for over a month without evidence of deterioration.

3. Metaphosphoric acid (reagent grade).   A 7 per cent aqueous solution is prepared fresh each day.

4. Zinc lactate (Eimer and Amend).   Prepare a stock standard solution by dissolving 165 mg. of $Zn(C_3H_5O_3)_2 \cdot 3H_2O$ in 500 cc. of water.   This gives a concentration (expressed as lactic acid) of 40 γ per 0.2 cc.   Dilute this as needed to give standards in the range of 2 γ to 10 γ per 0.2 cc.   Store in a cold room.

Apparatus—

1. Pyrex glass test-tubes, $\frac{5}{8} \times 5$ inches, equipped with ground glass stoppers.   To prevent the stoppers from blowing off when the tube is heated, the tube and stopper are provided with small glass arms around which rubber bands are wound.

2. Photoelectric colorimeter, equipped with a combination of filters, Wratten No. 21 and No. 61.

Procedure

Use 0.5 cc. of whole blood or plasma.   Precipitate the proteins according to Miller and Muntz (1938).   Carbohydrates, if present in concentration greater than 200 mg. per cent, yield yellow oxidation products which interfere with the determination.   To avoid this, the procedure of Peters and Van Slyke (1932) may be followed for the precipitation of protein and the subsequent removal of carbohydrate by the use of copper and lime.   This method is also satisfactory for the removal of 3 per cent gum acacia.   After the precipitation of the protein and carbohydrate 0.2 cc. samples ·of.the supernatant solution are taken for analysis.   Usually a thin scum is present on the surface of the liquid even after long centrifuging.   Before introduction of a pipette, this should be removed completely by touching with a glass stirring rod and again centrifuging if necessary.   It is extremely important that no particles of precipitate be sucked up in the samples taken for determination.   The analysis of these samples proceeds according

to the directions of Miller and Muntz with the following two exceptions. (1) In place of the addition of dry p-hydroxy-diphenyl, add 0.02 cc. of the reagent (in NaOH solution) with a Krogh micropipette. The reagent at first adheres to the glass but can be completely suspended in the solution by shaking for a few minutes. (2) After 1 hour, as described, heat in boiling water for 30 to 40 seconds (instead of 90), *agitating constantly.*

Solutions may be read in a photoelectric colorimeter. The colorimeter must be calibrated by making readings on a series of standard zinc lactate solutions (0.5 to 10 γ per 0.2 cc.) carried through the entire procedure. The weakest standard solutions, containing 0.5 γ per 0.2 cc., deteriorate in a few days. Stronger solutions, containing 8 γ per 0.2 cc., show a change after a month but none after 6 days.

By this method, the mean percentage difference between dupli-cate determinations on twenty-five samples of a perfusion solution containing 3 per cent gum acacia and 10 per cent dog cells in frog-Ringer's solution was 3.35 per cent. The standard deviation of these differences from the mean difference was ± 2.7. The percentage recovery of acid added to such a mixture varied between 92 and 99 per cent.

Remarks

The intensity of color produced by this modification is approxi-mately 88 per cent of that produced by the original method. The effect of this difference appears to be negligible in so far as accuracy and ability to determine small concentrations are concerned

No reason can be suggested for the fact that the optimum amount of p-hydroxydiphenyl is 0.45 mg. (0.02 cc.) when added in solution, while 8 mg. is the optimum amount for dry addition. Small amounts (about 1 mg.) added dry produce only about 40 per cent as intense a color as that given by 8 mg.

The utility of the ground glass stoppers is much less than might be supposed. The mean of fourteen determinations of a standard of 5 mg. per cent lactic acid with the stoppers left off during the heating was 4.74 ± 0.021 as compared to 4.99 ± 0.016 for twenty-five similar determinations with stoppers in place during the heat-ing. That is, there is only a 5 per cent loss of acetaldehyde when the stoppers are omitted. The probable errors of the two means

show, however, that the difference is probably significant.   Mendel and Goldscheider (1925), using a somewhat similar colorimetric procedure, but without stoppers in their tubes, found no loss of acetaldehyde.

Since acetone even in small amounts affects the reaction by causing a yellowish tinge, it should not be used for drying glassware. Pipettes may be dried safely by alcohol and ether.

A series of determinations was made of a standard lactic acid solution (5 mg. per cent), with varying amounts of the color reagent.   The optimum amount was found to lie between 0.010 and 0.025 cc.; 0.02 cc. was selected as an average.   Larger amounts give less color and with 0.4 cc., which contains 8 mg. of p-hydroxydiphenyl in solution, there results only a large amount of white precipitate and no color whatever either after the material has stood for 1 hour or on subsequent heating.

A series of determinations of a standard lactic acid solution (5 mg. per cent) was made, with varying lengths of time between the addition of the color reagent and the final heating.   There was no significant increase in color intensity after 45 minutes, and the color remained unchanged for at least 3 hours.   1 hour was selected as a safe minimum time.

After standing for 1 hour with the color reagent added, the solution still contains a certain amount of solid material which might interfere with the colorimeter reading.   This material can be dissolved by continual agitation in boiling water for 30 to 40 seconds as called for in the method.   The color of the solution is not significantly affected by heating times up to 90 seconds, but is diminished in intensity by more prolonged heating.

SUMMARY

The method for lactic acid determination described by Miller and Muntz (1938) was modified by introducing the color reagent, p-hydroxydiphenyl, in a solution of 0.18 M NaOH instead of in the dry form.   The average percentage difference (and standard

deviation) between duplicate determinations on 0.5 ml. of blood or blood plasma was 3.3 ± 2.7.

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BUFFER INFLUENCE ON TAKA-DIASTASE

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In studies on the relationship between activity and pH, reported for various enzymes, it has been observed by some investigators (1–7) that the pH optimum depends in part on the buffer employed to control the pH. Other workers, however, have found no evidence (8–13) of a shift in the optimum pH of enzyme activity with a change in buffer. The literature contains reports of still other specific buffer effects; namely, the relative activity of the enzyme at the optimum pH and on the acid and alkaline sides of the optimum as a function of the concentration and nature of the buffer cation, anion, and free acid. In much of this work the buffer mixtures of Sørensen, Walpole, McIlvaine, Palitzsch, Clark and Lubs, Cohn, and others have been commonly used.

Recent observations by Smith (14), Moyer and Abramson (15), and Davis and Cohn (16) that the ionic strength, as well as the nature of the ions present, is an important factor in determining the apparent isoelectric point of a protein suggest that enzyme activity measurements at different pH values would be more comparable if a constant ionic strength were maintained. Incidentally, Wisansky (17) has recently presented data from which he concludes that, at constant ionic strength, the influence of the neutral buffer acid concentration on the activity of invertase is also quite appreciable. At the pH optimum and at constant ionic strength, maintained by the addition of potassium chloride, an increase in total acetate buffer concentration was found to decrease the activity of the invertase, an effect which was attributed to the increase in neutral acetic acid concentration. Wisansky concluded that the decrease in activity observed with decrease of pH was due to the increased concentration of undissociated acid, but the evidence advanced is not sufficient to

exclude a possible concomitant specific effect of the acetate ion.[1] The findings of Hahn (19) that neutral salts inhibit the action of malt diastase only on the acid side of the pH optimum may be of interest in this respect.

In studies on malt amylase Sherman, Caldwell, and Boynton (12) found very little change in activity at the pH optimum when the concentration of acetate buffer was varied from 5×10^{-5} M to 0.10 M. Lövgren (20) and Van Slyke and Zacharias (21) reported that increase of the total phosphate buffer concentration did not shift the pH optimum for urease but caused a parallel decrease in activity, an effect similar to that found by Wisansky with acetate buffer and invertase, except that constancy of ionic strength was not maintained in the work with urease.

In the present communication we wish to report on the relationship between activity and pH in the case of taka-diastase and nine different buffers: formate, acetate, propionate, butyrate, valerate, phenyl acetate, phthalate, succinate, and citrate. A constant ionic strength of 0.05 was maintained for each buffer over the pH range considered. Further studies on the influence of buffer mixtures of constant ionic strength on the pH optimum and activity of β-amylase and invertase are in progress.

<div align="center">EXPERIMENTAL</div>

Preparation of Buffers—For the monobasic acids the concentration of the sodium salt was maintained at 0.05 M and the free acid concentration was varied to obtain the desired pH. The correct proportions of sodium hydroxide and buffer acid for the maintenance of constant ionic strength over the pH range with

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[1] Wisansky's conclusions rest largely on the data presented in Figs. 1 and 2 of his paper. As he himself recognizes, the increase in concentration of neutral acid which is represented in these figures is accompanied, necessarily, by an increased concentration of acetate ion. That an increase in acetate ion content above pH 4.6 is without effect on the activity of invertase is, in our judgment, of little significance. It is only below pH 4.6 where the acetate ion and the enzyme are of opposite charge (18, 14) that the acetate ion can be expected to affect specifically the activity of the enzyme. According to our conception of enzyme action, any inhibitory effect of the acetate ion would become progressively greater with increase of net positive charge on the enzyme; that is, as one proceeds to lower and lower pH values.

the di- and tribasic acids were deduced by combining the mass action expressions for the dissociation of the acid hydrogens, the equation for ionic strength, and the ion concentration relationships. For the dibasic acids the following general equations were obtained which express the total acid anion concentration, C, and the final concentration of the metal cation, in this case Na^+, as functions of the hydrogen ion activity, a_{H^+}, and the ionic strength, μ.

$$C = \mu\left[\frac{a_{H^+}^2(f_1/k_1) + a_{H^+} + k_2(f_1/f_2)}{a_{H^+} + 3k_2(f_1/f_2)}\right] \tag{1}$$

$$[Na^+] = \mu\left[\frac{a_{H^+} + 2k_2(f_1/f_2)}{a_{H^+} + 3k_2(f_1/f_2)}\right] \tag{2}$$

k_1 and k_2 are the dissociation constants for the acid hydrogens, f_1 and f_2 are the activity coefficients for the mono- and divalent acid anions, the values for which were approximated from the Debye-Huckel expression, $-\log f_i = 0.506\ (Z_i^2\ \sqrt{\mu}/1 + \sqrt{\mu})$.

Similarly Equations 3 and 4

$$C = \mu\left[\frac{a_{H^+}^3(f_1/k_1k_2) + a_{H^+}^2(1/k_2) + a_{H^+}(f_1/f_2) + k_3(f_1/f_3)}{a_{H^+}^2(1/k_2) + 3a_{H^+}(f_1/f_2) + 6k_3(f_1/f_3)}\right] \tag{3}$$

$$[Na^+] = \mu\left[\frac{a_{H^+}^2(1/k_2) + 2a_{H^+}(f_1/f_2) + 3k_3(f_1/f_3)}{a_{H^+}^2(1/k_2) + 3a_{H^+}(f_1/f_2) + 6k_3(f_1/f_3)}\right] \tag{4}$$

for tribasic buffer acids were obtained. The fundamentals involved in Equations 3 and 4 are not essentially different from those considered by Green (22) in her directions for the preparation of phosphate buffer mixtures of known ionic strength and pH. However, some difficulty was encountered in predicting the proportions of citric acid and sodium hydroxide necessary for a desired pH from the dissociation constants given in the literature.

Three parameters, also designated as apparent dissociation constants, for use in Equations 5 and 6

$$C_{total\ citrate} = 0.05\left[\frac{a_{H^+}^3(1/k'_1k'_2) + a_{H^+}^2(1/k'_2) + a_{H^+} + k'_3}{a_{H^+}^2(1/k'_2) + 3a_{H^+} + 6k'_3}\right] \tag{5}$$

$$[Na^+] = 0.05\left[\frac{a_{H^+}^2(1/k'_2) + 2a_{H^+} + 3k'_3}{a_{H^+}^2(1/k'_2) + 3a_{H^+} + 6k'_3}\right] \tag{6}$$

between pH 4 and 6 were calculated from the three simultaneous linear equations obtained by substituting in Equation 7

$$k \left[a_{H^+}^2 \left(\frac{C}{[Na^+]} - 1 \right) \right] + p \left[a_{H^+} \left(2\frac{C}{[Na^+]} - 1 \right) \right] +$$

$$q \left[3\frac{C}{[Na^+]} - 1 \right] = a_{H^+}^3 \qquad (7)$$

(in which $k = k'_1$, $p = k'_1 \times k'_2$, and $q = k'_1 \times k'_2 \times k'_3$) three sets of values for known mixtures of citric acid and sodium hydroxide; the resultant pH was measured with a glass electrode. Equation 7 was derived from the mass action expressions for the three apparent dissociation constants, k'_1, k'_2, and k'_3, and the ion concentration relationships, with no assumptions being made about the ionic strength. The known values for the proportions of acid and base were not arbitrarily chosen, but were those predicted from the general Equations 3 and 4, with the values for k_1, k_2, and k_3 given in the literature, and the actual pH of the solutions.

The process was then repeated by using this first set of parameters in Equations 5 and 6. Again three sets of values and hence three simultaneous equations were obtained from Equation 7, yielding a second set of parameters. Employment of this second set of parameters in Equations 5 and 6 gave relatively good agreement between the desired pH and measured pH when the predicted proportions of citric acid and sodium hydroxide were mixed.

When the values given in the literature of $k_1 = 8.7 \times 10^{-4}$, $k_2 = 1.8 \times 10^{-5}$, $k_3 = 4 \times 10^{-6}$, and the approximations of $f_1 = 0.81$, $f_2 = 0.43$, and $f_3 = 0.15$ were substituted in Equations 3 and 4, the results given in the upper sections of Table I were obtained.

For the first set of parameters, $k'_1 = -1.10 \times 10^{-2}$, $k'_2 = -1.12 \times 10^{-3}$, and $k'_3 = 2.80 \times 10^{-5}$, Equations 5 and 6 yielded the results shown in the middle section of Table I.

For the second set of parameters, $k''_1 = -2.12 \times 10^{-4}$, $k''_2 = 2.71 \times 10^{-4}$, and $k''_3 = 4.68 \times 10^{-5}$, substituted in Equations 5 and 6, the most satisfactory agreement was obtained (see Table I, lower section).

At any pH between 4 and 6 it can be shown that the relationship between the calculated concentrations of the mono-, di-, and

trivalent anions and the concentration of sodium ion required by the ionic strength equation, $\frac{1}{2}[C_1 + 4C_2 + 9C_3 + [Na^+]] = 0.05$, is approximately satisfied. The contribution of $[H^+]$ to the ionic strength was neglected. No attempt has been made to explain the significance of the negative parameters used in the above equations. It is obvious from this method of determining these parameters for Equations 5 and 6 that they can only be used to predict the correct proportions of acid and base for citrate buffer mixtures which have an ionic strength of very nearly 0.05. The above results would indicate that a redetermination of the thermodynamic dissociation constants for citric acid is to be desired.

TABLE I

Citrate Buffer Mixtures of Desired pH and Ionic Strength

Desired pH	Predicted $C_{total\ citrate}$	Predicted $[Na^+]$	Measured pH
	M	M	
4.00	0.0327	0.0399	2.40
5.00	0.0120	0.0282	4.18
6.00	0.00870	0.0253	5.54
4.00	0.0130	0.0300	4.13
5.00	0.00958	0.0262	4.83
6.00	0.00848	0.0251	5.70
4.00	0.0135	0.0305	4.10
5.00	0.00920	0.0258	5.05
6.00	0.00842	0.0251	6.03

Preparation of Substrate-Buffer Mixtures—The substrate-buffer mixture for a given buffer and pH was prepared in the following manner. In a 100 cc. volumetric flask calibrated for delivery were placed the calculated amounts of buffer acid and 1.0 N NaOH which on dilution to 100 cc. would yield the desired pH and ionic strength. The concentrated buffer ingredients were then diluted with 75 cc. of soluble starch solution containing 2 gm. of dry Lintner soluble starch (Merck). The starch solution was prepared in like manner for each run; *e.g.*, the proper weight of air-dried starch corrected for moisture content was dispersed in redistilled water by boiling for 15 minutes, cooled, and made up to volume. The buffer-starch solution was diluted to 100 cc. and placed in the thermostat at 30° for 2 hours.

Preparation of Reaction Mixture—25.0 mg. of taka-diastase (Parke, Davis and Company) were dissolved in several cc. of redistilled water and diluted to 25 cc.   For each run 1 cc. of enzyme solution containing 1 mg. of enzyme powder was pipetted into a 250 cc. Florence flask.   Zero time for the reaction was recorded when the 100 cc. of buffer-starch solution were poured into the reaction flask containing the enzyme.   The flask was swirled several times and then fixed in the thermostat.   At noted time intervals 5 cc. aliquots were withdrawn and the reducing power was determined by the Willstätter-Schudel hypodiodite method (23) as applied by Blom, Bak, and Braae (24).   Additional runs with maltose as the substrate showed a negligible increase in reducing power after several hours.   Thus it was assumed that maltase impurities in the enzyme had little effect on the initial rate of increase in reducing power when starch was the substrate.

Results

Fig. 1 presents the activity-pH curves obtained with formate, acetate, propionate, butyrate, and valerate buffers.   The reciprocal of the time for the reduction of 2 cc. of 0.05 N I_2 by a 5 cc. aliquot of digest (equivalent to the production of about 13 per cent of the theoretical maltose) is plotted on the ordinate axis as a measure of the activity.   Another index of enzyme activity frequently employed is the reducing power of the digest after a given time interval.   When the number of cc. of 0.05 N I_2 reduced by a 5 cc. aliquot of reaction mixture after 30 minutes of digestion was plotted against pH, curves very similar to those in Fig. 1 were obtained, thus demonstrating that either method yields similar results when brief periods of digestion are considered.
The activity-pH curves for phenyl acetate, phthalate, succinate, and citrate buffers are presented in Fig. 2.
An inspection of the activity-pH curves reveals that the optimum pH region, 4.9 to 5.4, is approximately the same for seven of the buffers.   A slightly more alkaline region, 5.1 to 5.5, is obtained with phthalate and citrate.   The relative saccharogenic activity of the enzyme at pH 5.1 and on the alkaline side of this optimum is not significantly different from buffer to buffer.   On the acid side of the optimum, however, an appreciable difference in the rate of production of reducing substances is evident and seems to depend on the buffer used.   In Fig. 1 it is interesting

to note the influence of carbon chain length of the buffer acid on the saccharogenic activity of the enzyme.

If, as in the case of malt amylase (25), the pH optimum of taka-diastase coincides with the isoelectric point, the variation in activity on the acid side of the optimum may be due to the association of the amphoteric enzyme molecule with the oppositely charged buffer anions of variable size, affinity, and specific influence. It is also possible that this difference in activity may be attributable to a specific action of the free buffer acid on the enzyme; at pH 4.0 the concentrations of acetic, propionic, butyric, and valeric acids are approximately 0.23, 0.30, 0.28, and 0.26 M.
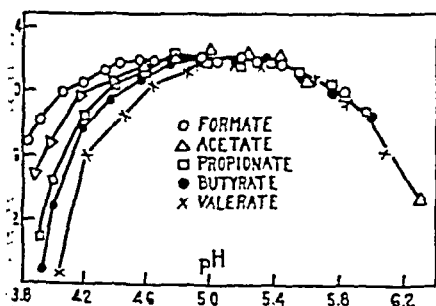
FIG. 1. Activity-pH curves with the reciprocal of the time for the reduction of 2 cc. of 0.05 N I_2 as the index of activity.
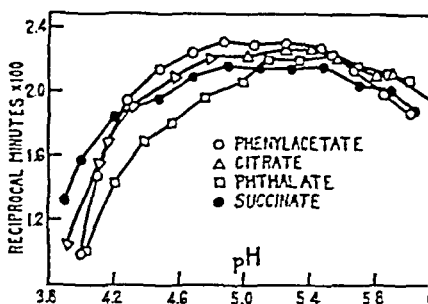
FIG. 2. Activity-pH curves with the reciprocal of the time for the reduction of 2 cc. of 0.05 N I_2 as the index of activity.

Formic and phenylacetic acids have higher dissociation constants and are considerably more dilute at this pH. However, if such a specific effect of the buffer acid were a factor, one might expect to find some variation in activity at the pH optimum for the different buffer acids. The work of Sherman, Caldwell, and Boynton (12) and Howell and Sumner (4) would indicate that the buffer acid has little influence on the enzyme when it is present in reasonably low concentrations.

SUMMARY

1. At 30° and an ionic strength of 0.05 the saccharogenic action of taka-diastase was found to have an optimum pH of 5.1 in the presence of formate, acetate, propionate, butyrate, valerate,

phenyl acetate, and succinate buffers.   An optimum pH of 5.4 was obtained when phthalate and citrate buffers were employed.

2. A variation of the buffer anion was without influence on the relative activity of the enzyme at the pH optimum and on the alkaline side of the optimum, but on the acid side marked differences in activity were observed.

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THE DETERMINATION OF TUNGSTEN IN BIOLOGICAL MATERIALS*

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State of South Carolina, Charleston)

In an investigation of the toxicity of several tungsten compounds
it was necessary to make accurate determinations of small quanti-
ties of tungsten in various biological materials. The method
finally evolved is described in this paper.

Principle

Organic material is destroyed by heating with a mixture of
sulfuric, nitric, and perchloric acids according to a modification
of the method of Barth (1) (as quoted by Smith (2)). The tung-
sten color reactions of Fer'yanchich (3) and Feigl and Krumholz
(4) have been modified and adapted to the use of the Evelyn
photoelectric colorimeter (5).

Reagents—

1. 1:1 HNO_3 (concentrated)-$HClO_4$ (70 to 72 per cent) mixture.
2. H_2SO_4, concentrated.
3. 40 per cent NaOH.
4. 15 per cent potassium thiocyanate.
5. HCl, concentrated.
6. Titanium trichloride reagent. Boil 1.0 cc. of 15 per cent
$TiCl_3$ with 2.0 cc. of concentrated HCl to expel traces of H_2S;
then dilute to 80 cc. with concentrated HCl. This reagent should
be prepared daily unless stored in completely filled glass-stoppered
bottles.
7. Stock tungsten solution (1.0 cc. $\backsimeq$ 1.0 mg. of W). Dissolve
1.261 gm. of tungsten trioxide in 100 cc. of 0.5 N NaOH and dilute
to 1000 cc.

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8. Standard tungsten solution (1.0 cc. ≃ 0.02 mg. of W). To 10.0 cc. of the stock tungsten solution add 50 cc. of 0.5 N NaOH and dilute to 500 cc.

Procedure

The method is applicable to urine or blood and other tissues. The procedure described below is based upon quantities of approximately 2.0 cc. of blood, 10.0 cc. of urine, or 2.0 gm. of organ. Any size sample may be used provided the volumes of the acid reagents are altered accordingly.

The sample is heated gradually in a 500 cc. Kjeldahl flask with several glass beads, about 4 cc. of concentrated H_2SO_4, and approximately 10 cc. of HNO_3-$HClO_4$ mixture. If charring occurs, HNO_3-$HClO_4$ mixture is added in small portions until the solution clears. Heat is gradually increased until the solution becomes concentrated and all dense white fumes are expelled from the flask.[1] After the flask cools, the acid residue is diluted with water and made distinctly alkaline to litmus with 40 per cent NaOH. The solution is boiled vigorously for several minutes, cooled, then transferred to a volumetric flask of such capacity that a 5.0 cc. aliquot will contain not more than 0.1 mg. of W. After dilution to the mark, the solution is filtered through a quantitative filter paper to yield a clear, colorless filtrate.

A 5.0 cc. aliquot (or other aliquot diluted to 5.0 cc.) is measured into the colorimeter absorption tube. 1.0 cc. of 15 per cent potassium thiocyanate, 4.0 cc. of concentrated HCl, and 1.0 cc. of titanium trichloride reagent are added with shaking. After 10 minutes, the tubes are placed in the colorimeter and read against a blank tube containing reagents only. Light Filter 420 (transmission 380 to 460 mμ) is used. The tungsten content is then obtained by reference to a calibration chart prepared from standard tungsten solutions varying in concentration from 0.0 to 0.1 mg. of W.

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[1] Substances such as bone and feces require relatively more H_2SO_4 than do other materials. The high salt content in these cases makes the final stage of digestion more difficult to recognize. However, no difficulty will be encountered in any case if all nitric and perchloric acid fumes are driven off so that sulfuric acid alone remains.

Recovery

Table I lists results obtained in experiments in which known quantities of tungsten were added to various biological materials.

TABLE I

Recovery Experiments

Substance	No. of experiments	Tungsten added	Tungsten found	Recovery
		mg.	*mg.*	*per cent*
5.0 cc. dog blood.......	1	0.0	0.0	
5.0 " " "	3	1.0	0.994, 0.994, 1.03	99.4, 99.4, 103
5.0 gm. rat liver.... .	1	0.0	0.0	
5.0 " " "	3	2.0	1.98, 1.95, 2.0	99.0, 97.5, 100
0.35 " " spleen ..	1	0.0	0.0	
0.55 " " "	3	0.5	0.51, 0.51, 0.506	102, 102, 101.2
1.31 " " kidney ..	1	0.0	0.0	
1.30 " " "	3	5.0	5.06, 5.1, 5.06	101.2, 102, 101.2
10.3 " " hind leg...	1	0.0	0.0	
9.45 " " " " .	1	10.0	10.585	105.8
3.0 " " gastrointestinal tract.	1	0.0	0.0	
" "	3	0.8	0.814, 0.808, 0.814	101.7, 101, 101.7
10.0 cc. human urine. .	1	0.0	0.0	
10.0 " " "	3	1.5	1.44, 1.44, 1.48	96, 96, 98.7
5.0 gm. rat feces......	1	0.0	0.0	
5.0 " " "	3	3.0	2.92, 2.92, 2.85	97.3, 97.3, 95

SUMMARY

A method for the determination of tungsten in biological materials is described. The range of recoveries is from 95 to 105.8 per cent, with an average recovery for the series of 99.9 per cent.

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FAT METABOLISM AFTER LIVER INJURY*

DECREASED FATTY ACID UTILIZATION BY MALE RATS FOLLOWING THE ADMINISTRATION OF CARBON TETRACHLORIDE

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During a previous investigation (1) tissue, fecal, and food fatty acids were estimated incidental to iodine number determinations. When balance calculations were made, it was found that the fatty acids which were "lost," or could not be accounted for (and which were assumed to be oxidized or otherwise converted by the organism), were very much smaller in amount in animals with liver damage than in normal rats kept under the same conditions. The present report deals with a confirmation and extension of this observation.

Procedure

Four groups of animals were studied. Each group was divided into subgroups of equal size, one of which was left untreated, while the members of the other were injected subcutaneously every other day of the experimental period with 0.05 cc. of carbon tetrachloride per 100 gm. of body weight. This dose of the drug was shown by histological study to produce extensive necrosis of the liver parenchyma, and is of the same order of magnitude as that employed by Cameron and Karunaratne in their study of the production of liver damage with carbon tetrachloride in the rat (2).

Male albino rats were used.[1] Groups I and II consisted of

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[1] The animals used for Groups I, II, and III were obtained from a local (Dallas) source. Those in Group IV were 80 to 100 gm. Sprague-Dawley rats obtained directly from Sprague-Dawley, Inc., Madison.

twelve animals each, Group III of twenty-four, and Group IV of 50. Group I was studied in July, the animals being kept at ordinary room temperature. Group II was observed in December to check possible seasonal influence. Groups III and IV were kept in constant temperature rooms. All animals of Groups I to III were allowed food *ad libitum*, while Group IV was subjected to a paired feeding experiment, the food intake of the normal animals being restricted to that of the treated rats. The experimental period for each group was 14 days.

To serve as a means of calculating the fatty acid content of the animals at the beginning of the experimental period, a comparable control group was killed and the per cent of body fatty acids determined at the start of each experiment.

The diet[2] used contained approximately 10 per cent of fatty acids, having an average iodine number of 41.

Methods

The methods of extraction and analysis used for Groups I to III have been reported (1). In general, the fatty acids were extracted by successive treatment with alcohol and ether, the fatty acids thus extracted being estimated by the method of Bloor (3). The bodies and feces of these groups were analyzed separately. This procedure was originally adopted in order to assure the extraction of unchanged fatty acids for iodine number determinations.

A different method was used for Group IV. The feces were not collected and extracted separately, but a procedure was adapted from that described by Schoenheimer and Breusch (4). The experimental animals were kept in individual Pyrex jars with removable wire platforms to prevent coprophagy. At the end of the experimental period, the wire platforms, food containers, and uneaten food were removed and the animals, without removal from the jars, were killed with ether. Into each jar (containing a killed animal, all of its feces, and spilled food) 300 cc. of alcohol and 150 gm. of potassium hydroxide were placed. All material soluble in alcoholic potassium hydroxide was dissolved

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[2] Ground Tioga dog food plus 7 per cent coconut oil, as described previously (1). In the diet for Group IV, Purina Dog Chow was substituted for Tioga dog food.

by boiling for 1 hour on a water bath. The resulting solutions were washed through glass wool into 500 cc. volumetric flasks, cooled, and made to volume with alcohol. 25 cc. aliquots were taken, alcohol was removed by evaporation under reduced pressure on the water bath, and, after dilution with water and acidification with 10 N sulfuric acid, the samples were extracted for 6 hours with petroleum ether in continuous liquid extractors. The petroleum ether was removed completely and the residual material weighed. This material was then dissolved in 30 cc. of the Bloor alcohol-ether mixture, 1.0 cc. of 10 per cent digitonin in alcohol added, and the mixture evaporated almost to dryness, during which process the sterol present in the extract was precipitated as the digitonide. 15 cc. of distilled water were added and the mixture heated for several minutes on the water bath. The flasks were then removed, 25 cc. of acetone added to facilitate filtration as suggested by Yasuda (5), and the contents of the flasks filtered through tared sintered glass crucibles. The precipitate of digitonide was washed three times each with acetone and ether, dried, weighed, and the sterol content of the aliquots calculated. By subtracting the sterol value from the weight of the total extract the amount of "fatty acid" present in each aliquot was determined. The values for fatty acids and sterol determined in this manner represented the amount of these substances present in the bodies of the experimental animals at the end of the period plus the amount extracted from the feces. The amount of fatty acid and sterol present in the spilled food also appeared here, but since these values occurred in the food eaten as well, they were canceled in the calculation of the fat loss.

The method of extraction and analysis described above has given on repeated trials recoveries of added fatty acids ranging from 95 to 104 per cent, and of added cholesterol from 97 to 107 per cent. Duplicate analyses on aliquots of the same alcoholic potassium hydroxide extract check within 2 per cent, while duplicate extractions and analyses of the same sample check within 5 per cent for total extract.

The fatty acid and sterol content of the control rats (killed at the start of the experiment) and of the diet fed the experimental animals of Group IV was determined by extraction with alcoholic potassium hydroxide and analyses as described above.

Calculation of Fatty Acid Loss—The percentage of fatty acids in the bodies of the control groups sacrificed at the start of each experiment was used to calculate the amount of fatty acid present in the bodies of both normal and treated animals when first placed on the experimental diet. To this value was added the amount of fatty acid ingested. From this sum the amount of fatty acid found in the feces and in the bodies of the animals at the end of the period was subtracted. This calculation was carried out on each animal, the resulting individual values for fatty acid loss being averaged for the normal and for the treated animals. In addition, the average gain or loss of sterol of both normal and treated rats of Group IV was found by similar calculations. Finally, the results obtained from the largest group (No. IV) were treated statistically. The standard errors of the means and of the differences of the means of this group were determined by the usual methods.

Results

In each individual calculation the amount of fatty acid found at the end of the period was less than the fatty acids of the body at the start plus the food fatty acids. It is assumed that the lost fatty acids calculated as described above were oxidized or otherwise converted to other substances by the organism during the experimental period.

The data obtained from Groups I to III are given in Table I, those from Group IV in Table II.

It will be seen that subsequent experiments confirmed the original observation (Group I) of a decreased fatty acid "loss" in the presence of liver damage, although in no other case was such a large difference between the normal and treated animals found. The average fatty acid loss of the treated animals was less than that of the normal animals whether the observations were made in summer (Group I) or winter (Group II), at ordinary room temperature (Groups I and II) or in constant temperature rooms (Groups III and IV), or whether both treated and normal animals were allowed free access to food (Groups I, II, and III) or paired feeding was instituted (Group IV). The significance of this decreased fatty acid loss in the presence of liver damage is attested by the constancy of its occurrence in four different series

TABLE I

Fatty Acid Loss in Rats with Food Unrestricted; Alcohol-Ether Extraction

The figures are given in gm.

	No. of rats	Body weight at start	Food fatty acids	Body fatty acids at end	Total fecal fatty acids	Fatty acid loss	Gain in body weight
Group I. Diet, 12.9 per cent fatty acids; 6 control rats, 4.61 per cent fatty acids							
Normal......	6	143	15.33	14.36	0.97	6.60	22
Treated.....	6	146	12.01	16.01	0.91	1.83	−7
Group II. Diet, 10.1 per cent fatty acids; 6 control rats, 8.69 per cent fatty acids							
Normal......	6	101	11.86	6.98	1.19	12.75	16
Treated.....	6	91	10.09	7.68	1.29	9.08	11
Group III. Diet, 11.5 per cent fatty acids; 12 control rats, 9.01 per cent fatty acids							
Normal......	12	118	14.50	10.89	1.02	13.22	21
Treated.....	12	109	12.91	11.29	1.29	10.75	8

TABLE II

Fatty Acid Loss in Group IV (Paired Feeding); Alcoholic KOH Extraction

Diet, 13.03 per cent fatty acids, 0.196 per cent sterol; average intake per rat for the period, fatty acids 12.1 gm., sterol 182 mg.; twenty-eight control rats, 3.93 ± 0.21 per cent fatty acids, 0.265 ± 0.004 per cent sterol.

	No. of rats	Body weight at start	Body plus feces at end		Fatty acid loss	Sterol gain or loss	Gain in body weight
			Fatty acids	Sterol			
		gm.	gm.	mg.	gm.	mg.	gm.
Normal......	25	86	6.45	425	9.00 ± 0.18	$+16 \pm 7.6$	6 ± 2.0
Treated.....	25	90	7.58	379	7.74 ± 0.21	-42 ± 8.0	2 ± 2.5
Difference....................					1.26 ± 0.28	58 ± 11.0	

of experiments and by the fact that in a group large enough to permit statistical evaluation (Group IV) the observed decrease was 4.5 times its standard error.

The treated animals in the three groups the normal rats of which were allowed food *ad libitum* showed a much reduced gain in body weight as compared to the normals (Table I). This effect is correlated with the reduced food intake of the poisoned animals, for the normal rats of Group IV, with a food intake restricted to the level of the treated animals, showed no significant increase in gain in weight as compared to the treated animals (Table II).

TABLE III

Group I. Liver Fatty Acids per 100 Gm. of Body Weight

	Rat No.	Liver weight	Total fatty acids	Phospholipid fatty acids
		gm.	*mg.*	*mg.*
Normal rats	1	2.97	197	65
	2	2.81	144	67
	3	2.54	165	50
	4	2.85	161	54
	5	2.91	140	48
	6	2.72	148	48
Average..........................		2.80	159	55
Treated rats	7	4.41	460	56
	8	4.49	478	91
	9	3.85	377	57
	10	5.59	392	51
	11	3.93	460	51
	12	4.31	338	62
Average..........................		4.43	418	61

When the bodies of the experimental animals were analyzed separately (Groups I, II, and III), it is seen (Table I) that the body fatty acids of the treated animals were larger in amount at the end of the experiment than were those of the normal rats of the same groups. This increase in body fatty acids cannot be wholly accounted for by the increased amounts of fatty acids found in the livers of the treated animals (Table III).

The findings as regards the sterol metabolism of Group IV are significant. It will be seen in Table II that slightly more sterol was recovered from the normal animals than could be accounted for in the food ingested and the bodies of these animals at the start of the experiment. Although the increase found in the normal rat

is probably not significant, the treated animals show a definite loss, or disappearance, of sterol. This increased loss is significant when compared with the increase in the normal animals, the difference being more than 5 times its standard error.

DISCUSSION

The data presented in this paper show that rats poisoned with carbon tetrachloride have a deranged fat metabolism which is manifested by a decrease in the amount of fatty acid loss—that is, fatty acid which cannot be accounted for in balance experiments. Since it has been shown (2) that carbon tetrachloride attacks primarily the liver, it is probable that liver damage is the cause of the observed decrease in fatty acid oxidation or conversion, and therefore that this oxidation or conversion of fatty acids to other substances must take place in whole or in part in the liver. It has been reported that carbon tetrachloride treatment causes a decreased iodine number of tissue, fecal (1), and plasma (6) fatty acids. It is suggested that the reduced fatty acid oxidation or conversion as a result of liver damage as found here is related to this interference with desaturation mechanisms resulting from liver damage.

The findings in Group IV show that carbon tetrachloride poisoning, and therefore liver damage, produces a definite disturbance of sterol metabolism. The increased loss found after liver damage may be accounted for by a decreased synthesis or an increased destruction of sterol, or both. The data presented here do not indicate which, if either, explanation is correct. Cholesterol synthesis has been shown to occur in the mouse (4), while a decrease in serum cholesterol (especially cholesterol esters) is a common finding in hepatic disease.

Effect of Carbon Tetrachloride Administration on Liver Lipids— The total and phospholipid fatty acids were determined in the alcohol-ether extracts of the livers of Group I by the methods of Bloor (3, 7) and are listed in Table III. The increase in fatty acids in the livers of the treated animals is comparatively slight when compared to the degree of fatty infiltration resulting from such procedures as high cholesterol or high fat diets and yet is of the same order of magnitude as that reported by Barrett *et al.* (8) as occurring in rats treated with carbon tetrachloride. The increased fatty acids found in the livers of the poisoned rats

reported here account for only 15 per cent of the increase in total body fatty acids noted over the normal values. The significant finding noted here is that in the presence of a disturbance of fat metabolism which results in a decreased fatty acid oxidation or conversion, an interference with fatty acid desaturation mechanisms, and a decreased sterol synthesis or increased destruction or both, all of which are apparently due to liver damage, the liver phospholipids are not significantly changed in amount.

The author wishes to express his appreciation of the encouragement and patient criticism of Professor L. A. Crandall, Jr., which have been invaluable aids in the preparation of this report.

SUMMARY

1. The fatty acid balance of male albino rats fed a diet containing 10 per cent of fatty acids (iodine number 41) was determined over a period of 14 days. In each case the disappearance or "loss" of fatty acids was noted.

2. When liver damage was produced by carbon tetrachloride treatment, the fatty acid loss was less than in normal animals kept under similar conditions.

3. Carbon tetrachloride treatment causes an increased loss of sterol.

4. This treatment causes no alteration in the amount of phospholipid fatty acid present in the livers of rats during the 2 week period used in this study.

5. It is concluded that liver damage produces a change in fat metabolism which results in a decreased oxidation or other conversion of fatty acids and an increased destruction or decreased synthesis of sterol.

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CHEMICAL DETERMINATION OF THIAMINE BY A MODIFICATION OF THE MELNICK-FIELD METHOD*

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Recently Melnick and Field (1) published an excellent article on the chemical determination of thiamine (vitamin B_1) wherein they adapted the Prebluda-McCollum (2) diazotized reagent to a quantitative basis, measuring the depth of red color produced by the vitamin B_1. In this report we have tested this general procedure with respect to what influence some of the other vitamins and complex mixtures might have. Their method was followed except in the adsorption and elution steps for which a somewhat simpler technique was introduced. Also we substituted the Lovibond tintometer in place of the colorimeter, in the belief that it permits a wider scope for the depth of color. In general, as the following data will show, our findings confirm those of Melnick and Field, the results being in close agreement with the theoretical and bioassay values.

EXPERIMENTAL

Standard Curve—In using the Lovibond tintometer, we first ascertained the degree of accuracy for known solutions of varying concentration of crystalline thiamine. The data are given in Table I. It is seen that as the concentration per 3 cc. of test solution ranges from 5, 10, 20, and up to 50 micrograms of thiamine, the red units increase in direct proportion, giving for all practical purposes a complete quantitative response. This enabled us to construct a standard curve (Fig. 1) from which to calculate the red units to micrograms of thiamine.

Application of Method to Thiamine Preparations—Pure thiamine

* Presented before the meeting of the American Society of Biological Chemists at New Orleans, 1940.

chloride in tablet form and in sterile solutions was assayed. The results are given in Table II. The respective values for the samples represent the average of three to five determinations each.

TABLE I

Determination of Thiamine by Melnick-Field Method, with Lovibond Tintometer

Thiamine per 3 cc.	Tintometer reading (red units)	Thiamine found	Per cent recovery
γ		γ	
5	1.08	4.8	96.0
10	2.21	10.2	101.0
20	4.26	20.09	100.4
30	6.26	29.79	99.3
40	8.43	40.26	100.6
50	10.68	50.15	100.3
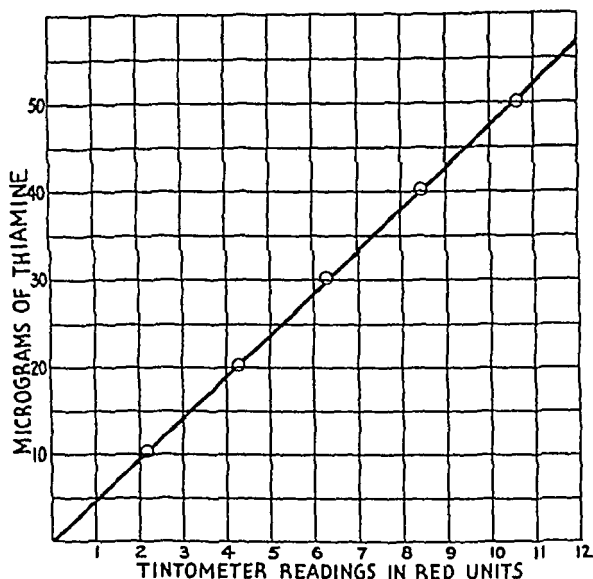
FIG. 1. Standard curve of reference for thiamine

Determination of Thiamine Combined with Other Vitamins—
Next, it was of interest to apply the method to various combinations of thiamine with such synthetic vitamins as riboflavin, vitamin B_6,[1] nicotinic acid, pantothenic acid,[2] and ascorbic acid.

[1] Supplied by Merck and Company, Inc.
[2] Courtesy of Dr. T. H. Jukes.

TABLE II

Determination of Thiamine in Special Preparations

Sample No.	Product	Chemical assay	Deviation from mean	Theoretical potency	Per cent of theoretical
		units per cc.	*per cent*	*units per cc.*	
33,519	Ampules	372	+4.0 to −2.2	367	101.3
32,249	Tablets	372*	+4.8 " −4.3	367*	101.3
29,939	Ampules	1,047	+1.9 " −2.3	1,100	95.2
33,469	"	2,217	+2.0 " −2.3	2,200	100.8
33,109	Vials	8,948	+2.8 " −2.2	8,667	103.2
34,540	"	20,322	+5.0 " −3.1	20,000	101.6

* Units per tablet.

TABLE III

Determination of Thiamine in Presence of Other Vitamins

Riboflavin (Vitamin B₂)	Pyridoxine (Vitamin B₆)	Nicotinic acid	Pantothenic acid	Ascorbic acid	Thiamine recovered (30 γ thiamine as starting material)
γ	γ	γ	γ	γ	*per cent*
					99.3
20					98.2
100					98.8
200					92.5
	20				102.5
	200				96.3
		100			99.3
		500			99.8
		1000			98.8
		3000			98.8
			100		100.5
			300		101.5
				50	94.7
				500	92.6
				1000	91.0
				2000	0.0
20	20				102.7
200	200				96.3
20	20	100			98.8
200	200	500			98.2
20	20	100		50	97.5
200	200	500		500	91.8

Except when ascorbic acid was present at high levels such as 2000 micrograms, the percentage recovery of thiamine was close to the theoretical (Table III).

Determination of Thiamine in Presence of Vitamin C—We found that the interference of ascorbic acid could be overcome to a great extent by one of two procedures: First by removing a substantial part of the vitamin C by adding cautiously either 0.01 N iodine solution or 1.0 per cent $K_3Fe(CN)_6$ solution and then following with the regular method for thiamine. However, if too much of either reagent is used, the vitamin B_1 is attacked and the

TABLE IV

Assay of Thiamine in Presence of Ascorbic Acid

Series	Thiamine	Ascorbic acid	Special treatment	Recovery of thiamine	Per cent recovery
	γ	γ		γ	
A	30	Control		29.8	99.3
B	30	500	None	27.8	92.7
	30	1000	"	27.3	91.0
	30	1000	1.04 cc. 0.01 N iodine	30.8	102.7
	30	1000	1.15 " 0.01 " "	27.8	92.7
C	30	2000	None	0.0	0.0
	30	2000	1.15 cc. 0.01 N iodine	16.3	54.3
	30	2000	2.08 " 0.01 " "	24.6	82.0
	30	2000	2.3 " 0.01 " "	21.9	73.0
D	30	2000	0.2 " 1% $K_3Fe(CN)_6$	0.0	0.0
	30	2000	0.4 " 1% "	23.4	78.0
	30	2000	0.6 " 1% "	28.8	96.0
	30	2000	0.7 " 1% "	29.8	99.3
	30	2000	0.9 " 1% "	7.1	23.7
	30	2000	1.3 " 1% "	0.0	0.0
E	20	2000	Adsorption method	19.4	97.0
	30	2000	" "	26.2	87.3
	40	2000	" "	36.3	90.8

value will then be too low. Second, in order to overcome the depressing effect of ascorbic acid, adsorb the vitamin B_1 on superfiltrol[3] as stated in detail later. In Table IV, the values for the iodine titration are given in Series B and C, for the cyanide in Series D, and for the adsorption in Series E. The data bring out the above facts clearly.

[3] Manufactured by the Filtrol Corporation, Los Angeles.

Direct Determination of Thiamine in Biological Preparations—
The direct method was next applied to some soluble preparations
which contained appreciable amounts of protein or sugars, or other
foreign matter along with free thiamine.   The results in Table V
include the bioassay data.   The values given for the chemical
assay are the averages of three to five tests.   With the exception
of the fortified liver extract, the data agree closely with the
biological values.

*Adsorption Method for Determining Thiamine in Biological
Mixtures*—In some instances the vitamin may either be in a
complex soluble mixture or in a combined form as in yeast where
it is often necessary to digest the material to split off the vitamin.
In either case the interfering factors must be eliminated.   This
has been accomplished in the main by adsorption of the thiamine
on superfiltrol.   Our detailed method is outlined as follows:

Dilution of Sample—Make up a stock solution of the test
material of such concentration that it contains approximately 5
to 20 micrograms of thiamine per each cc.   Take varying amounts,
i.e. 2, 3, 4 cc., in 15 cc. centrifuge tubes and dilute each to 5 cc.
with distilled water.

Adsorption—Adjust to pH 4 to 5 with dilute HCl.   Add 0.1
to 0.15 gm. of superfiltrol.   Shake occasionally during the next
hour.   Centrifuge and discard the supernatant.

Reaction of Thiamine with Prebluda-McCollum Reagent—Add
to the adsorbate 3 cc. of water and 3 cc. of 95 per cent alcohol
containing 5 mg. of phenol per cc.   Add 1 drop of thymol blue and
adjust the reaction to pH 7 to 8 with dilute NaOH.   Add 6 cc.
of the alkaline diazotized reagent (2).   Mix thoroughly with the
adsorbate.   Allow to stand 2 hours or more at room temperature.
Filter on a small Hirsch filter.   Wash the adsorbate with about
5 cc. of water.

Elution Stage—Transfer the adsorbate and paper to a dry cen-
trifuge tube.   Add 2 cc. of 95 per cent alcohol.   Stopper and shake
to dissolve the pigment.   Centrifuge, decant the solution into a
tintometer tube, and read on the Lovibond tintometer.

Calculations—Evaluate in terms of thiamine from the standard
curve, taking an average of the results obtained from the three
assays.

The results from the application of the adsorption procedure

TABLE V

Determination of Thiamine in Biological Mixtures

Sample No.	Product	Chemical assay		Biological assay	Per cent of biological
		Mean value	Deviation from mean		
		units	*per cent*	*units*	
34,180	Elixir	98.6 (Per cc.)	+2.7 to −5.7	100 (Per cc.)	98.6
34,100	Wheat germ extract	44.3 (" gm.)	+2.3 " −1.4	43.3 (" gm.)	102.3
33,059	Emulsion vitamins A, B, D	285.2 (" cc.)	+0.87 " −0.77	285 (" cc.)	100.0
34,440	Liver extract, fortified	256 (" fluid oz.)	+9.7 " −6.6	224 (" fluid oz.)	114.3
28,869	Stomach extract with vitamin B_1	53.1 (" capsule)	+15.0 " −8.4	57 (" capsule)	93.2

for the quantitative determination of thiamine are shown in Table VI. Here the potency is given for thiamine itself and for

TABLE VI

Determination of Thiamine in Biological Samples by Direct and Adsorption Methods

Sample No. and product	Bioassay value	Amount taken for assay	Direct method		Adsorption method	
	units per mg.	γ	*units per mg.*	*per cent**	*units per mg.*	*per cent**
16,709. Thiamine standard	333.3	20	334.1	100.2	332.2	99.7
		30	333.8	100.1	331.3	99.4
		40	339.2	101.8	337.9	101.4
			335.7	**100.7**	**333.8**	**100.2**
	units per cc.	*cc.*	*units per cc.*		*units per cc.*	
34,900. Yeast concentrate	217	0.04	206	94.9	218	100.5
		0.05	191	88.0	204	94.0
		0.06	183	84.3	230	105.9
		0.08	163	75.1	222	102.3
			186	**85.6**	**218**	**100.7**
	units per gm.	*gm.*	*units per gm.*		*units per gm.*	
35,120. Dried brewers' yeast	57	0.1	32.0	56.1		
		0.15	20.4	35.8		
		0.2	16.1	28.2	58.5	102.6
		0.25	7.9	13.8	58.1	101.9
		0.3			58.4	102.4
		0.35			57.0	100.0
			19.1	**33.5**	**58.0**	**101.7**
31,359. Wheat germ extract	40	0.2	42.1	105.3	41.5	103.8
		0.4	24.9	62.3	40.5	101.3
			33.5	**83.8**	**41.0**	**102.5**
	units per fluid oz.	*cc.*	*units per fluid oz.*		*units per fluid oz.*	
34,720. Elixir	160	1	124	77.5	149	93.1
		2	118	73.8	154	96.2
			121	**75.6**	**151.5**	**94.7**

The figures in bold-faced type represent the averages.
* Expressed in per cent of the bioassay values.

four different types of materials, by (*a*) the biological method, (*b*) the direct chemical method, and (*c*) the adsorption-chemical method.

As was to be expected, the standard thiamine gave, by the adsorption procedure, as good a recovery of vitamin as by the direct method. With the three complex mixtures such as soluble yeast concentrate (Sample 34,900), wheat germ extract (Sample 31,359), and elixir mixture (Sample 34,720), the values obtained by the direct method were not only lower than those for the bioassay method but they decreased as the amounts taken for assay increased. On the other hand, by the adsorption method, the values obtained for all levels agreed closely and within the practical limits of the biological tests.

When the vitamin was in the combined form as in dried yeast (Sample 35,120), the material was digested with a mixture of takadiastase and papain, an extract made, and the vitamin B_1 adsorbed from the resulting solution with superfiltrol. The data for the direct and adsorption methods show that the latter gave results agreeing closely with the bioassay value.

In summation, we have modified somewhat the Melnick-Field chemical method of determining thiamine quantitatively by substituting the Lovibond tintometer for the colorimeter, and by replacing the complicated permutit adsorption and elution steps with a simpler procedure with superfiltrol.

We confirm the authors' observations that when the Prebluda-McCollum diazotized reagent is used with thiamine, it is possible to adapt it to a quantitative method. We find (1) riboflavin, nicotinic acid, pyridoxine (vitamin B_6), and pantothenic acid do not interfere even when present in comparatively large amount. (2) Ascorbic acid in excess does interfere, however, giving very low values. This can be overcome by adsorption with superfiltrol. (3) In general with simple and complex mixtures, the method gives quantitative results that are in conformity with the data obtained by bioassay methods.

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THE FORM IN WHICH ACETONE BODIES ARE PRODUCED
BY THE LIVER

By LATHAN A. CRANDALL, Jr.

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Jowett and Quastel (1) have shown that surviving liver slices, respiring in a medium containing fatty acid as a substrate, produce roughly 40 per cent of acetone bodies in the form of β-hydroxybutyric acid and 60 per cent as acetone plus acetoacetic acid. But as far as the author is aware, no one has attempted to determine which of the acetone bodies are added to blood by the liver of the intact animal. This observation is of interest as providing some indication of the path followed by this type of fat metabolism.

Unanesthetized angiostomized dogs were used, the methods being similar to those previously employed in other studies on such animals (2). The dogs were fasted, or subjected to a period of fasting followed by the oral administration of 100 ml. of olive oil per day. Total acetone bodies were determined by the method of Crandall (3), the acetone plus diacetic acid fraction being estimated separately by the same technique but without the addition of potassium dichromate and the period of refluxing being shortened to 40 minutes. β-Hydroxybutyric acid was taken as the difference between the total acetone bodies and acetone plus acetoacetic acid. The validity of this method of determining β-hydroxybutyric acid was checked in several instances by hydrolysis of acetoacetic acid with sulfuric acid and removal of the resulting as well as preformed acetone by boiling. The remaining β-hydroxybutyric acid was then determined in the usual manner. The results of such direct determination of β-hydroxybutyric acid checked with determinations by difference within the limits of error of the method.

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Data on the arterial blood acetone body concentration, per cent of the total which is β-hydroxybutyric acid, the hepatic output of acetone bodies, and the per cent of the hepatic output that is β-hydroxybutyric acid are given in Table I.   All data are expressed as mg. of β-hydroxybutyric acid per 100 ml. of blood.   The common practice of expressing acetone body concentrations as acetone is believed to be less informative, since β-hydroxybutyric acid usually constitutes more than 50 per cent of the total.   The hepatic output is calculated, as in previous studies on hepatic me-

TABLE I

Form in Which Acetone Bodies Are Added to Blood by Liver

The hepatic output is calculated by the formula, hepatic venous concentration minus ($\frac{3}{4}$ portal venous concentration plus $\frac{1}{4}$ arterial concentration).   The hepatic outputs of total acetone bodies and of β-hydroxybutyric acid were calculated separately.

Dog No.	Fasted	Fat fed	Total acetone bodies		β-Hydroxybutyric acid as per cent of total acetone bodies	
			Arterial concentration	Hepatic output	Arterial blood	Hepatic output
	days	*days*	*mg. per 100 ml.*	*mg. per 100 ml.*		
1	11	0	8.1	5.5	88	93
2	11	0	4.7	4.2	94	79
3	6	9	31.4	6.6	51	56
4	6	7	12.2	6.8	65	35
4	6	9	40.0	5.7	71	62
1	11	7	11.6	15.9	72	72
2	11	6	9.6	5.4	74	56
3	6	11	27.2	6.9	64	70

tabolism by the angiostomy method, by assuming that the portal blood contributes three-fourths and the hepatic artery one-fourth of the total inflow and subtracting a weighted average from the hepatic venous value.   Changes in the proportion of blood contributed by the portal vein and hepatic artery would affect the results slightly if at all, since the concentration of acetone bodies in arterial blood is but slightly higher than that in the portal vein. It should be noted that when the proportion of β-hydroxybutyric acid in the arterial blood is low, it is also low in the hepatic output, and when high in arterial blood tends to be a larger fraction of hepatic output.

Marriott (4) in 1914 showed that β-hydroxybutyric acid constitutes 52 to 79 per cent of the total acetone bodies in the blood of human diabetics in ketosis. In one dog reported by him β-hydroxybutyric acid amounted to 65 per cent of the total. The range for arterial blood in our dogs is slightly wider than that for man, since they show 51 to 94 per cent. Of particular interest is the fact that the proportion of β-hydroxybutyric acid added to the blood by the liver is substantially the same as the proportion of this substance in the circulating blood. It is known from the work of Marriott (4), Wilder (5), and others that acetoacetic acid is readily converted to β-hydroxybutyric acid in the body, but that the reverse process is difficult. It was anticipated that acetoacetic acid might be the form in which the greater part of the ketone bodies is liberated, and that the predominance of β-hydroxybutyric acid could be due to formation of this substance from acetoacetic acid by the extrahepatic tissues. This does not appear to be the case. Our results strongly suggest that the proportions in which the acetone bodies accumulate in the blood are approximately the same as those added by the liver and that the tissues must therefore use these substances in the same proportion. Crandall, Ivy, and Ehni[1] have previously found that not more than 5 per cent (and probably less) of the acetone bodies formed by the liver is eliminated in the urine of the dog. Renal elimination would therefore have little effect on the ratio present in the blood.

It is believed that the agreement between the per cent of the total hepatic output as β-hydroxybutyric acid in our experiments and the figure of 40 per cent β-hydroxybutyric acid formation found by Jowett and Quastel (1) for surviving liver slices is reasonably good; they term their estimate of the percentage "very rough," and it should be recalled that their data are from rat and guinea pig liver slices while our results were obtained on dogs.

SUMMARY

The proportions of β-hydroxybutyric acid and of acetone plus acetoacetic acid that are added to the blood by the liver are of

[1] Crandall, L. A., Jr., Ivy, H. B., and Ehni, G. J., unpublished data.

the same order of magnitude as those found in the circulating blood. β-Hydroxybutyric acid constitutes from 35 to 93 per cent of the total hepatic acetone body output and 51 to 94 per cent of the total arterial blood concentration in the dog. This is considered evidence that these substances are oxidized by the tissues in substantially these same proportions.

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THE DETERMINATION OF ACETONE AND ACETOACETIC ACID IN BLOOD BY THE BISULFITE-BINDING METHOD AND ITS RELATION TO PYRUVIC ACID

By DANIEL KLEIN

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Clift and Cook (1) have described a method for the determination of carbonyl compounds in blood as bisulfite-binding substances. Devised primarily for rapid determination of pyruvic acid, the procedure as described made possible the determination of acetoacetic acid to the extent of about 87 per cent, but acetone could not be measured because of the instability of the addition product. Sufficient research has been carried out by a number of workers to establish the proper conditions for the quantitative recovery of acetone in pure solutions as well as acetoacetic acid by the bisulfite addition reaction. Equilibrium studies with carbonyl-bisulfite compounds by Kerp and his collaborators are reported in some detail by Kolthoff *et al.* (2) and Parkinson and Wagner (3). Jolles (4) and Stepp and Engelhardt (5) also describe a somewhat unwieldy method for the estimation of large amounts of acetone as bisulfite-binding substances. Modifications of the Clift and Cook method have been reported (6–9), but none provides the conditions for completely stabilizing the acetone-bisulfite compound.

A distinct need exists for a simple method for the determination of ketone substances. By introducing modifications into the Clift and Cook procedure it has been possible to adapt it to the determination of acetone and acetoacetic acid in blood.

Reagents—

$N/12$ sulfuric acid solution.

10 per cent sodium tungstate solution.

25 per cent copper sulfate solution.

Powdered calcium hydroxide.

1 M sodium bisulfite solution.   Prepare fresh weekly and keep in a refrigerator.

1 per cent starch solution.   Prepare fresh every few days and keep in a refrigerator.

1 N iodine solution, approximate.   130 gm. of iodine and 125 gm. of potassium iodide in 1 liter of water.

0.1 N iodine solution, approximate.

0.005 N iodine solution.   Make by diluting 5 cc. of 0.1 N iodine to 100 cc.

Disodium phosphate, 12 H_2O, pulverized.

Procedure

Tungstic acid blood filtrates are made by the Haden modification (10) of the Folin-Wu method (11).   Glucose is removed as in the lactic acid determination (12): 0.1 volume of 25 per cent copper sulfate is added, followed by enough powdered calcium hydroxide to make the solution alkaline; the mixture is shaken at intervals for $\frac{1}{2}$ hour and then centrifuged.

10 cc. aliquots are allowed to react with 1 cc. of 1 M sodium bisulfite in stoppered 50 cc. Erlenmeyer flasks, and placed in a refrigerator at about 5° for $\frac{1}{2}$ to 2 hours.   0.5 cc. of 1 per cent starch solution is then added, and the excess bisulfite is removed by the addition successively of 1 N, 0.1 N, and 0.005 N iodine solutions.   The first end-point is reached when a purple or blue color remains for at least 30 seconds.   With small amounts of ketones this end-point lasts much longer.   The total volume at this stage is approximately 15 to 16 cc.   The bound bisulfite is released by the addition of 2 gm. of disodium phosphate (6).   The released bisulfite, equivalent to the acetone present, is titrated with 0.005 N iodine until a stable purple or blue color is attained.   The calcium phosphate which precipitates does not interfere with the determination.

Blank determinations with distilled water either with or without copper-lime treatment show a titer of from 0 to 0.04 cc. of iodine.   1 per cent tungstic acid solutions made according to the Folin-Wu-Haden procedure under the same conditions yield blanks which are much greater than the values found in normal blood. Pure tungstic acid solution cannot therefore be used as a blank.

The interfering substances causing the high blanks are removed with the blood protein precipitate.

1 cc. of 0.005 N iodine is equivalent to 0.145 mg. of acetone. When the sample represents 10/11 cc. of blood (10 cc. of Folin-Wu filtrate freed of sugar), the calculation becomes (mg. of acetone per 100 cc. of blood) = 15.95 $(A - B)$, where A = cc. of 0.005 N iodine to titrate bisulfite bound by acetone and B = cc. of iodine used in the blank.

TABLE I

Recovery from Blood of Added Acetoacetic Acid As Bisulfite-Binding Substances

Room temperature, 20-26°; temperature of refrigerator, 4-6°. Acetoacetic acid calculated as acetone.

Acetoacetic acid added	Amount recovered from sugar-freed Folin-Wu filtrates of blood		Acetoacetic acid added	Amount recovered from sugar-freed Folin-Wu filtrates of blood	
mg. per 100 cc.	mg. per 100 cc.	per cent	mg. per 100 cc.	mg. per 100 cc.	per cent
180.4	170.0	94.2	4.5	3.9	86.7
	168.8	93.6		4.7	104.4
180.4	178.1	98.7	4.5	4.2	93.3
	175.7	97.4		4.2	93.3
135.3	136.5	100.7	3.4	3.4	100.0
	134.5	99.4		3.4	100.0
90.2	89.7	99.5	3.4	2.9	85.3
	83.6	92.7		3.4	100.0
45.1	46.4	102.9	2.3	2.3	100.0
	43.5	96.5		2.1	91.3
6.7	5.8	86.6	2.3	2.1	91.3
	6.3	94.0		2.4	114.3

Recovery of Acetone and Acetoacetic Acid—Recovery experiments with acetoacetic acid and acetone are reported in Tables I and II. The acetone, reagent quality, was weighed and diluted, and by the Van Slyke method (13) showed 100 per cent purity. The Van Slyke gravimetric procedure was used also to determine the concentrations of the added acetoacetic acid, prepared according to Shaffer (14). Duplicate determinations by the bisulfite-binding method show excellent agreement. Complete recovery of acetone is obtained from aqueous solutions before and after copper-lime treatment. Recoveries of acetone from blood, after removal of protein and sugar, are less complete especially in the higher

concentrations of acetone than are the excellent recoveries of acetoacetic acid. Loss by adsorption and evaporation during the course of preparing the blood filtrate may be the cause. Since in ketonemia acetoacetic acid is present to a much greater extent than acetone, actually loss of acetone does not impair appreciably the usefulness of the method.

TABLE II

Recovery from Aqueous Solutions and Blood of Added Acetone As Bisulfite-Binding Substances

Room temperature, 20–26°; temperature of refrigerator, 4–6°.

Acetone added	Acetone recovered					
	Aqueous solutions				Blood, Folin-Wu filtrates freed of sugar	
			Copper-lime treatment			
mg. per 100 cc.	mg. per 100 cc.	per cent	mg. per 100 cc.	per cent	mg. per 100 cc.	per cent
290.5	287.6	99.0	285.1	98.1		
	291.3	100.3	282.4	97.2		
145.3	143.9	99.0	145.9	100.4	106.1	73.0
	148.0	101.9	145.7	100.3	105.4	72.5
145.3					120.5	82.9
					119.8	82.5
72.6	72.0	99.2	73.0	100.6	63.7	87.7
			71.8	98.9	62.6	86.2
72.6					64.5	88.8
					62.9	86.6
29.1	29.9	102.7	29.1	100.0	24.4	83.8
	29.3	100.7	28.8	99.0	24.3	83.5
29.1					26.4	90.7
					26.0	89.3
17.5					16.3	93.1
					16.3	93.1
11.6					11.3	97.4
					11.2	96.6
5.8					6.3	108.6
					6.3	108.6

For comparison with these recoveries a number of determinations were carried out by a slight modification of the Van Slyke gravimetric procedure. The changes consisted in the use of small volumes of Folin-Wu filtrates freed of sugar with proportionally smaller amounts of sulfuric acid and mercuric sulfate

solutions, and the washing of the precipitate with hot instead of cold water to remove all the tungstate. Recoveries were of the

TABLE III

Simultaneous Determination in Blood of Pyruvic Acid and Total Carbonyl Compounds (Bisulfite-Binding Substances)

All values are expressed as mg. per 100 cc. 1 mg. of pyruvic acid is equivalent to 0.66 mg. of acetone.

Patient No.	Pyruvic acid	Copper-lime treatment		
		Pyruvic acid	Bisulfite-binding substances as acetone[*]	Minus pyruvic acid
Normal patients				
1. Overnight fasting..........................	0.55	0.49	1.7	1.4
2. " "	0.85	0.77	1.6	1.1
3. " "	0.59	0.59	1.0	0.6
1 hr. post prandial..........................	0.78		0.6	
4. 48 hrs. fasting..........................	0.79		2.7	
13 " "	1.04		1.1	
140 " "	0.83		9.4	
5. Overnight fasting..........................	0.85		1.3	
Patients on diets low in thiamine				
6. 1 hr. post prandial..........................	1.97		1.3	
7. Overnight fasting..........................	1.32		1.7	
8. " "	1.27		0.6	
9. " "	1.47	0.77	1.6	1.1
10. " "	0.70		0.6	
" " 10 days later on same diet..........................	0.96	0.70	1.9	1.4
Diabetic patients				
11. Overnight fasting, controlled..............	1.06	0.93	1.6	1.0
12. 14 hrs. after admission in acidosis.........	2.64	1.25	16.2	15.4
13. 52 " " " " "	0.68		10.4	

[*] Duplicate determinations agreed within 0.04 cc. of 0.005 N iodine.

same order as in the bisulfite-binding method. The results with both methods compare favorably with those obtained by recent modifications of the Van Slyke procedure (15–18).

Bisulfite-Binding Substances and Pyruvic Acid—Pyruvic acid is also estimated as bisulfite-binding substances (1). The present procedure was employed to ascertain the fate of pyruvic acid subjected to copper-lime treatment. After removal of the sugar, 55.2 per cent was recovered from an aqueous solution containing 11.7 mg. per cent of pyruvic acid. When added to blood, 3.2 and 6.4 mg. were recovered to the extent of 112 and 90.2 per cent before and 43.0 and 47.5 per cent respectively after copper-lime treatment. These concentrations when found in blood are abnormally high. In order to study the effect of copper-lime treatment on pyruvate present in blood, the dinitrophenylhydrazine method was utilized (19, 20).[1] The pyruvate was stabilized with iodoacetate (20). Total bisulfite-binding substances and pyruvic acid were determined simultaneously (Table III). Of the values reported there are seven in which pyruvate was estimated both before and after removal of the sugar. With the two abnormal concentrations, 1.47 and 2.64 mg., approximately 50 per cent was removed by copper-lime treatment. Of the remaining specimens with normal values, from 0 to 27 per cent was destroyed.

DISCUSSION

It has been claimed that determination of bisulfite-binding substances could be used to measure the pyruvic acid level of blood, cerebrospinal fluid, or urine (21–24) and thus to evaluate the degree of thiamine deficiency. Thompson and Johnson (21) were able to demonstrate a correlation between blood bisulfite-binding substances and pyruvate in polyneuritic pigeons. In thiamine deficiency produced experimentally in animals, others have also reported the successful application of the Clift and Cook method (23, 24), chiefly because the vitamin deficiency was the major variable. In applying the method clinically Platt and Lu (25), who previously had reported favorable results (22), and others (7–9) concluded that the determination of bisulfite-binding substances is not an accurate measure of pyruvate in blood or cerebrospinal fluid. Our results indicate that the Clift and Cook procedure merely approximates variations in total carbonyl substances in body fluids and unless it can be definitely established

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[1] Klein, D., unpublished data.

that only pyruvic acid is present deductions concerning this substance are not justified.

The bisulfite-binding capacity of glucose is small on account of the very large dissociation constant of glucose-bisulfite, $K_{25°} = 2.2 \times 10^{-1}$ (2), yet appreciable owing to the relatively high concentration of glucose. That this error due to glucose is not constant can be concluded from the wide range of normal values for bisulfite-binding substances in human blood obtained by the Clift and Cook method (7, 8, 22, 25). These variations may be caused by a number of factors according to the mass action law; e.g., concentration of glucose, temperature, and reaction time. The effect of fluctuations in glucose concentration is manifested by the following. It has been observed by us and others (1, 6) that bisulfite-binding substances due to glucose are roughly proportional to the amount present. That the normal amount of bisulfite-binding substances in blood filtrates from which sugar had not been removed is higher than that of cerebrospinal fluid filtrates (8) appears to be due to the fact that blood sugar concentrations are higher than those of cerebrospinal fluid.

The experimental results demonstrate that the bisulfite-binding method described will quantitatively determine acetone and acetoacetic acid in ketonemia of widely varying degree. The acetone-bisulfite compound is unstable at room temperature because of its large dissociation constant, $K_{25°} = 4 \times 10^{-3}$ (2). It has been shown by our results that the acetone-bisulfite reaction is carried to completion and stabilized by (a) allowing sufficient time for the addition reaction, (b) lowering the temperature, (c) keeping the total amount of added solution to a minimum during the course of removal of excess bisulfite. Any other carbonyl compound in the filtrate freed of sugar forming a bisulfite addition product with a dissociation constant equal to or smaller than that of acetone-bisulfite will also be completely recovered.

When pyruvate exists in normal amounts in blood, the bisulfite-binding substances determined as acetone, in filtrates freed of sugar, include most of this acid. With high pyruvic acid values, the method detects about 50 per cent of the pyruvate originally present in the blood.

That this method is useful in disclosing variations in ketonemia can be observed by the acetone levels obtained with Patients 4, 12,

and 13 (Table III). The values for carbonyl compounds expressed as acetone in normal blood here reported are of the same order as those found by others (16, 18). For the determination of the normal range this procedure is probably not as accurate as the aforementioned modifications (16, 18) of the Van Slyke mercuric sulfate method with the omission of the dichromate oxidation to exclude β-hydroxybutyric acid. However, these mercuric sulfate precipitates besides measuring acetone may include other carbonyl compounds (26); *e.g.*, pyruvic acid. That the latter does precipitate under the conditions employed in the Van Slyke procedure has been confirmed by us. Thus the bisulfite-binding method is no less specific for acetone and acetoacetic acid than is the Van Slyke procedure.

SUMMARY

A simple method for determining acetone plus acetoacetic acid depending on bisulfite-binding of carbonyl groups is described. These compounds are completely bound with bisulfite in Folin-Wu filtrates freed of sugar and addition products are stabilized in the presence of a large excess of sodium bisulfite at low temperatures.

Evidence is presented showing that this method can be used to determine blood carbonyl compounds, expressed as acetone.

The relation between bisulfite-binding substances and pyruvic acid is clarified. Since pyruvate is included in the values for the bisulfite-binding substances, its exact estimation should be carried out by the more specific dinitrophenylhydrazine procedure.

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THE ABILITY OF CITRULLINE TO REPLACE ARGININE IN THE DIET OF THE CHICK

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Earlier work (1) in this laboratory has shown that arginine is essential for maintenance as well as growth of the chick. Also, it was shown that ornithine, with or without urea, cannot replace arginine in the diet. The present work is a test of the ability of citrulline to duplicate the growth-promoting effect of arginine in casein diets.

The experiments were conducted similarly to those described previously (1). The newly hatched, single comb white Leghorn chicks were placed on a standard chick mash for 1 week, then weighed, wing-banded, divided into groups of the same average weight and weight distribution, and placed on the experimental diets. The chicks were weighed individually at 1 or 2 day intervals.

The arginine and ornithine used were commercial products. The citrulline was prepared from ornithine by the method of Kurtz (2). The basal diet, described in Table I, was not severely deficient in arginine, as the casein alone furnished 0.74 per cent of this amino acid to the diet. However, the increase in growth due to the addition of 1 per cent arginine hydrochloride to this diet is large enough to allow it to serve as a significant test for the arginine-replacing ability of any compound.

The results, given in Table II, show that citrulline is able to replace the supplementary arginine in its growth-promoting effect. It appears that the chick is capable of substituting an imino group for the oxygen in the ureido group of citrulline, thus converting it to arginine. The former finding that ornithine cannot replace arginine is confirmed. In one attempt to test ornithine dihydro-

chloride at higher levels it was found that a level of 2 per cent was detrimental, since it permitted an average growth rate of only 0.4 gm. per chick per day, much less than that of chicks on the basal diet.

TABLE I

Basal Diet

	parts		parts
Casein	20	Hexane extract of alfalfa..	≃2
Brewers' yeast	5	Cholic acid	0.25
Yeast extract	≃10	Sodium chloride†	1
Chondroitin*	2	Potassium chloride	0.5
Glycine	1	Calcium carbonate	1
Cystine	0.3	Tricalcium phosphate	3
Cod liver oil	1	Cotton pulp	5
Wheat germ oil	3	Cerelose (dextrose)	56

* Chondroitin was replaced by 5 per cent gum arabic in Experiment II.
† Plus traces of Mn, Fe, Co, Cu, Zn, Al, and I.

TABLE II

Relative Growth-Promoting Effects of Arginine, Ornithine, and Citrulline As Supplements to Chick Basal Diet

Supplement to basal diet	Average gain per chick per day	
	Experiment I*	Experiment II†
	gm.	gm.
None	2.3	2.9
1% arginine hydrochloride	5.5	5.1
1% ornithine dihydrochloride	2.0	3.2
1% citrulline	5.7	5.2

* Ten chicks were kept on the diet for 5 days in each case.
† Six chicks were kept on the diet for 10 days in each case.

The present results do not preclude the possibility that ornithine might have been utilized to a very slight extent. In our former report, however, it was shown that ornithine could not even retard the *loss* of weight of chicks on a diet much lower in arginine content than the basal diet used in these experiments. The amount of ornithine utilized must be very small, if any is used. Thus, even in the presence of arginase, which is notably absent

in the liver of the fowl, the ornithine cycle of Krebs and Henseleit (3) would be inoperative because of the chick's inability to convert ornithine to citrulline.

In a later experiment we tested the ability of creatine and guanidine to replace arginine. Entirely negative results were obtained.

SUMMARY

1. The ability of citrulline to replace arginine as a supplement to an arginine-low diet for the chick has been demonstrated.

2. The inability of ornithine to replace arginine effectively has been confirmed.

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KETOGENIC ACTIVITY OF ACETIC ACID

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It is well known (1–5) that acetic acid is rapidly utilized when fed to mammals. Whether it is oxidized directly or after conversion into other compounds is uncertain. Loeb (6) and Friedmann (7) have shown that acetic acid increases the yield of acetoacetic acid in the perfused isolated liver. The present study was designed to determine whether or not acetic acid is ketogenic in the intact organism.

The macro-Kjeldahl procedure was used for urine nitrogen determinations, Van Slyke's method (8) for the determination of urine acetone bodies, and the method of Barnes and Wick (9) for the determination of blood acetone bodies. Other methods are those which we have used before (10–12) in related studies.

After many unsuccessful trials a satisfactory experiment was carried out in which acetic acid was fed to a phlorhizinized dog. As Deuel and Milhorat (5) have noted, vomiting occurs as a usual thing after the administration of sodium acetate to dogs. Free acetic acid is even worse in this regard and only in this one experiment was emesis avoided. The cathartic action of the salt (5) is not shared by the free acid. The results in Table I show that when acetic acid is fed there is a striking increase in the excretion of acetone bodies in the urine. This was accompanied by a marked rise in the urine nitrogen excretion.

When acetic acid is fed to fasting rats (Table II), there is always an increase in the ketonuria. This is true whether the acetic acid is fed as such or partially or entirely neutralized. Blood acetone body determinations indicate that this increased urinary excretion is not a result of changes in the renal threshold but results from an increased production of acetone bodies. That

the blood acetone body levels of the acetic acid-fed animals are not greatly higher than the controls is due to the fact that the determinations were made 12 hours after the last feeding of the acid. With antiketogenic substances this time element is largely absent in so far as the decrease in the blood ketone level is concerned, for the antiketogenic action is due to the glycogen which is formed and this exerts a fairly constant action over a long period (12).

Acetic acid then is ketogenic in fasting rats as well as in the phlorhizinized dog. It is interesting in this regard that acetic

TABLE I

Influence of Feeding Acetic Acid on Urinary Excretion of Ketone Bodies in Phlorhizinized Dog

A canine bitch weighing 11.6 kilos was fasted for 10 days and observations made the last 6 days. It was given a subcutaneous injection of 10 cc. of 10 per cent phlorhizin suspended in olive oil every day. Urine specimens were collected by catheter. The acetic acid was fed by stomach tube in 3 per cent solution.

Length of period	Urine nitrogen	Urine ketones	Acetic acid fed during period
hrs.	*gm. per hr.*	*gm. per hr.*	*gm.*
24	0.347	0.043	
24	0.355	0.060	
20	0.663	0.195	10
24	0.784	0.242	20
25 .	0.490	0.122	10
15	0.408	0.080	
7	0.288	0.023	

acid causes a marked increase in the total metabolism (3) and Lusk (13) has observed that, "Just as carbohydrate and fat, when given together, increase the metabolism by the sum of the effects which either alone would produce, so between carbohydrate and acetic acid there was a summation of effect." There is some evidence that acetic acid may be utilized directly by the muscles. However, when acetone body production is taking place, it is probable that exogenous acetic acid is utilized by the organism after conversion to acetone bodies in the same manner as other fatty acids with an even number of carbon atoms. While the higher acids may reach this form by β oxidation, acetic acid must

TABLE II

Ketogenic Activity of Acetic Acid in Fasting Rats

Experiment No.	No. of rats*	Average body weight (gm.)	Average body surface (sq.dm.)	Solution fed in dose of 1 cc. per sq.dm. body surface	Urine N per sq.dm. body surface per day (mg.)	Urine acetone bodies per sq.dm. body surface per day†				Blood acetone bodies at end of			
						1st day (mg.)	2nd day (mg.)	3rd day (mg.)	4th day (mg.)	1st day (mg. per cent)	2nd day (mg. per cent)	3rd day (mg. per cent)	4th day (mg. per cent)
1	6	173	3.5	1.0 M NaHCO₃	30.5	2.7	5.0	3.0	2.7				
	6	173	3.5	1.0 " " + 0.5 M acetic acid	31.6	4.9	28.2	8.5	5.0				
2	3	199	3.9	0.75 " "	15.7	0.6	16.0	35.9	35.5				
	8	198	3.9	0.75 " " + 0.75 M acetic acid	16.4	17.4	50.6	54.4	51.2				
3	6	183	3.7	0.75 " "	18.3	7.1	12.3	24.9	25.5				
	6	185	3.7	0.75 " " + 1.0 M acetic acid	24.9	50.9	72.1	61.6	66.8				
4	4	151	3.2	0.75 " "	21.5	0.8	2.2	2.5					
	4	147	3.2	0.75 " " + 0.75 M acetic acid	26.7	2.9	27.2	28.1					
5	3	201	3.9	0.25 " NaCl	19.0	1.3	25.3	17.0	15.1	41	50		67
	3	198	3.9	0.25 " " + 0.25 M acetic acid	19.5	3.5	30.4	31.0	24.3	45	51		60
6	4	201	4.6	0.25 " "	16.0	15.3	18.9	13.6	10.8		47	40	45
	4	204	4.7	0.25 " " + 0.25 M acetic acid	10.9	15.9	20.2	30.9	20.0		46	49	43
7	6	237	4.3	Water	15.3	12.1	22.7	16.2	5.1		58	61	45
	6	237	4.3	0.25 M acetic acid	18.7	22.2	24.4	20.9	15.1		61	72	59
	6	237	4.3	0.25 " NaHCO₃ + 0.25 M acetic acid	15.3	75.2	51.0	39.2	20.4		66	68	59

* Male rats were used in Experiments 1, 6, and 7. Female rats comprised the other experiments.

† Two doses of the solutions listed were fed each day except in Experiments 5 and 6 in which three and four doses respectively were administered daily.

‡ The rats in Experiments 1 and 4 were fasted directly from the stock diet, while all of the rest received a low protein diet for 4 to 23 days prior to fasting.

be converted to acetoacetic acid by condensation. It is known (14) that in the form of its ester acetic acid may be condensed in the test-tube to the ester of acetoacetic acid.

The increase in nitrogen excretion produced in the phlorhizinized dog by feeding acetic acid occurs to a less extent in the fasting rat. It cannot be the result of an acidosis as is the increased protein catabolism produced by feeding inorganic acids, for acetic acid does not reduce the carbon dioxide-combining power of the blood (4). The effect of acetic acid on nitrogen metabolism may not be specific, but a property of other fatty acids as well. Data on this point are not available.

This demonstration that acetic acid may give rise to acetone bodies in the intact mammalian organism has an important bearing upon the current conception of the method by which fatty acids are oxidized in the organism under circumstances such that acetone bodies are being formed. Acetone bodies are produced in the liver and carried by the blood stream to the muscles and other tissues for final oxidation. The old conception of β oxidation with the production of only 1 molecule of acetone body from 1 molecule of even the long chain fatty acids is no longer tenable. On this basis an impossible amount of fat would have to be burned to furnish the quantity of acetone bodies which is known to be utilized during a ketosis as a result of studies (15) of the difference in the concentration of acetone bodies in the arterial and venous blood. In a study of the oxidation of fatty acids by isolated liver slices Jowett and Quastel (16) found a larger quantity of acetone bodies than successive β oxidation as usually considered would account for. Then Butts (17) and Deuel (18) and their coworkers observed in rat feeding experiments a far greater yield of acetone bodies from the higher fatty acids than Knoop's hypothesis would provide for. Blixenkrone-Møller (19) compared the oxygen consumption of perfused livers of diabetic cats with the total acetone body production and could explain the low oxygen-acetone body ratio only by assuming that 4 molecules of acetone body were formed per molecule of fatty acid. Recently Stadie, Zapp, and Lukens (20) have found that the molecular ratio of oxygen consumed to acetone bodies produced from fatty acids by liver slices is far from that which would be expected by classical β oxidation. These observations have led to the rise of

a theory for the production of acetone bodies by a mechanism other than β oxidation. Hurtley (21) first proposed this hypothesis of fatty acid oxidation in which the fatty acid is attacked at alternate carbon atoms simultaneously along the whole length of the chain. He rejected the old β oxidation hypothesis because he felt that if it were correct large amounts of the lower fatty acids intermediate between the long chain acids and the acetone bodies should be found in an intense ketosis. He overlooked the probability that these intermediate reaction compounds need only exist momentarily within the liver cells. Jowett and Quastel (16) adopted Hurtley's scheme of fatty acid oxidation which they called the "multiple alternate oxidation" hypothesis. Deuel *et al.* (18) propose a combination of the "multiple alternate oxidation" theory for the higher fatty acids with the usual β oxidation theory applied to caproic and butyric acids.

There is no direct proof of any kind to support the "multiple alternate oxidation" hypothesis as the method by which acetone bodies arise during the oxidation of fatty acids. There is plenty of evidence for the successive β oxidation of various compounds in metabolism (22). It is the most reasonable explanation of the formation of acetone bodies from caproic and butyric acids. With the evidence which has been presented in the experimental portion of this paper that acetic acid is an acetone body former the classical β oxidation hypothesis may be modified so that it may explain all of the findings which have been cited against it.

When a fatty acid is oxidized by β oxidation, 2 carbon atoms are dropped from the chain together. What form this cleavage takes is unknown but it is usually pictured as productive of acetic acid. If this were true and the acetic acid formed acetone bodies which we know it may do, there would be little difficulty in retaining the β oxidation hypothesis in the light of our present knowledge. All of the carbon atoms in a fatty acid molecule with an even number of carbon atoms could yield acetone bodies (1 molecule of $C_{16} \rightarrow 4$ molecules of acetoacetic acid; 2 molecules of $C_{18} \rightarrow 9$ molecules of acetoacetic acid; etc.) which would account for the high acetone body production which made the mechanism of β oxidation appear impossible (15, 17–19). Stadie *et al.* (20) support the multiple alternate oxidation hypothesis, because they find an observed molecular ratio of oxygen consumption to ketone

bodies produced in liver slices of 1.68:1 which is reasonably corrected to 1.54:1. They point out that this is not far from the theoretical ratio for palmitic acid, if submitted to multiple alternate oxidation, of 1.25:1. But it is much closer to the theoretical ratio of the modified β oxidation hypothesis which is 1.625:1. However, we must remember that rate observations of this kind, based upon mutilated bits of liver tissue in an abnormal environment, must be used with great care in drawing any quantitative conclusions. Because there was some unidentified fixed acid formed by liver slices during the oxidation of higher fatty acids, Jowett and Quastel (16) considered the possibility of acetone bodies being formed by β oxidation via acetic acid and discarded the idea because acetic acid produced acetone bodies at a slower rate than the higher fatty acids and because benzoate poisoning inhibited acetone body production by acetic (and butyric) acid but enhanced it for the higher fatty acids. Even though these observations had not been obtained from injured pieces of liver tissue which it is hard to believe may function normally, it would not appear necessary to discard the modified β oxidation hypothesis because of them.

The β oxidation hypothesis in its modified form should be retained until data are available which give better support of some other scheme. If it is correct, the acetic acid may never exist as such in appreciable amounts. For conditions under which extracellular (as far as the liver cells are concerned) acetone bodies are not required by the organism, that is, there is no ketosis, there is not even a need to suppose that the acetic acid is converted to acetone bodies. If β oxidation is the mechanism for the oxidation of fat when extracellular acetone bodies are not being formed, the acetic acid might be oxidized directly to carbon dioxide and water.

SUMMARY

When acetic acid is fed to a phlorhizinized dog or to fasting rats, there is an increase in the excretion of acetone bodies in the urine. Blood acetone body concentrations show that this is not due to changes in the renal threshold and the conclusion is reached that there is an increased production of acetone bodies. This is interpreted as a result of the conversion of acetic acid to acetoacetic acid.

Acetic acid feeding during fasting results in an increase in protein catabolism.

The formation of acetone bodies by acetic acid is used to support a modified view of the β oxidation hypothesis of fat metabolism which meets all of the important objections which have been raised to this hypothesis.

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A STUDY OF THE PERMEABILITY OF HUMAN ERYTH-ROCYTES TO POTASSIUM, SODIUM, AND INORGANIC PHOSPHATE BY THE USE OF RADIOACTIVE ISOTOPES*

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In 1927 Wakeman, Eisenman, and Peters (9) demonstrated that the human red blood cell behaves as if it were impermeable to potassium and sodium added *in vitro*. Upon addition of the salts of these elements to defibrinated blood, osmotic equilibrium between cells and serum was maintained by the passage of water alone out of the cells. These experiments have since been amply confirmed and extended in this laboratory and elsewhere. However, it cannot be concluded from experiments of this type that the red cell membrane is impermeable to sodium and potassium, since the possibility remains that a considerable interchange of base between cells and serum takes place across the cell membrane which cannot be demonstrated because the amount entering is exactly equal to the amount leaving.

By adding radioactive isotopes of these elements to blood it is possible to determine whether such equivalent interchange of individual atoms across the cell membrane takes place.

Experiments similar to those of Wakeman, Eisenman, and Peters were carried out with radioactive sodium, Na^{24}, and po-

tassium, K^{42}. The final concentrations of radioactive material in cells and serum were determined after equilibration, in addition to the chemical analysis.

A further series of experiments was carried out with inorganic phosphate containing P^{32}. Halpern (7) had previously made experiments on the penetration of inorganic phosphate into the red blood cell at various temperatures, using a technique similar to that of Wakeman, Eisenman, and Peters. It seemed desirable to repeat her experiments with radioactive phosphorus.

Methods

Male subjects without demonstrable disease affecting the red blood cells were used.[1] Potassium chloride, sodium chloride, and neutral sodium phosphate, prepared from the radioactive hydroxides and anhydride respectively, were the salts employed. Blood was defibrinated aerobically and divided into two portions, one serving as a control. In the other portion the cells were sedimented by centrifugation, the dried salt dissolved in the supernatant serum, and the cells recombined with the serum. Both this and the control specimens were drawn into glass bulbs containing a little mercury, air was excluded, and the two samples placed in an incubator at 38°. Every half hour for 4 hours the bulbs were agitated, the mercury serving to mix the cells and serum. The samples were then removed from the incubator and the hematocrits of each determined with Daland tubes. Part of each specimen was set aside for whole blood analyses, the rest was centrifuged and the serum drawn off for analysis.

Sodium and potassium were determined by Hald's modifications of the methods of Kolthoff and of Shohl and Bennett (6). Phosphate was determined by the method of Fiske and Subbarow (4).

Radio activity was determined by the use of a Geiger counter tube that dipped into the solution to be analyzed. Whole blood was hemolyzed with saponin and water. Both whole blood and serum were diluted with water, the dilutions being made so that each gave approximately the same actual count, avoiding the

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[1] The majority of the subjects had hypertension or coronary sclerosis. Several were slightly polycythemic. No diabetics were included.

need for a calibration curve. Counts were usually made over a 4 to 6 minute period and repeated at least once. Background was determined and subtracted from the total count. Radioactive decay was negligible with these isotopes in the time necessary for the whole counting to be finished. It is estimated that the accuracy of the method with practice is in the neighborhood of 5 per cent. In a few of the earlier experiments it probably did not exceed 7 or 8 per cent.

Calculations—Cell concentrations were obtained by the formula,

$$\text{Concentration in cells} =$$

$$\frac{\text{whole blood concentration minus (1 minus cell volume)} \times \text{serum concentration}}{\text{cell volume}}$$

Transfers were obtained by the formula,

$$\% \text{ transfer} =$$

$$\frac{\begin{array}{l}\text{whole blood concentration}_2 \text{ minus (1 minus cell volume}_2) \times \text{serum}_2 \\ \text{minus whole blood concentration}_1 \text{ minus (1 minus cell volume}_1) \\ \times \text{serum}_1\end{array}}{\text{whole blood concentration}_2 \text{ minus whole blood concentration}_1} \times 100$$

The subscripts 1 and 2 refer to the initial (or control) and final samples respectively.

All values so obtained are expressed in terms of cell and serum water. Average values were assumed for the initial cell and serum water (3), as follows: concentration$_1$ per unit of serum water = concentration$_1$ per unit of serum $\div$ 0.93; concentration$_1$ per unit of cell water = concentration$_1$ per unit of cell $\div$ 0.72.

Since the cells shrank markedly with addition of salt, these values could not be used for the final serum and cell values. It was assumed that only water passed, and the equations became

$$\text{Concentration}_2 \text{ per unit serum water} =$$

$$\text{concentration}_2 \text{ per unit serum} \div \left(1 - 0.07 \frac{(1 \text{ minus cell volume}_1)}{(1 \text{ minus cell volume}_2)}\right)$$

$$\text{Concentration}_2 \text{ per unit cell water} =$$

$$\text{concentration}_2 \text{ per unit cell} \div \left(1 - 0.28 \frac{(\text{cell volume}_1)}{(\text{cell volume}_2)}\right)$$

Radioactivities are obtained originally in impulses per minute, with the sample in the same fixed relation to the Geiger counter tube. The radioactivities of both whole blood and serum in impulses per minute are multiplied by that factor which will make the specific radioactivity per unit element in serum water unity. If complete simple equilibrium were established between the elements in cells and serum, the specific radioactivity in cells should be unity, whereas it would be zero if no penetration or exchange occurred.

TABLE I

Exchanges of Potassium between Cells and Serum

Experiment No.	Potassium added per liter whole blood	Specific radio-activity of added potassium	Concentration of potassium per liter water				Specific radio-activity of potassium in cells†	Transfers from serum to cells, per cent of amount added	
			Serum		Cells			Potassium, net	Radioactive potassium
			Initial	Final	Initial	Final			
	m.eq.	*units* per m.eq.*	*m.eq.*	*m.eq.*	*m.eq.*	*m.eq.*	*unit per m.eq.*		
1	61	5	5.3	100.2	136	213	−0.04	+3	−3
2	25	59	5.4	48.8	126	147	0.03	+2	+5
3	29	11	5.0	56.6	122	195	0.05	−4	+6
4	30	40	4.9	56.9	122	151	0.07	−1	+8
5	28	41	6.4	52.1	113	142	0.05	+7	+6

* Arbitrary units.

† The units are so defined in each experiment that the specific activity of the potassium in serum water is unity.

Results

Potassium—Five experiments with potassium are summarized in Table I. The specific radioactivity of cells after 4 hours ranged from −0.04 to +0.07, figures probably not significantly differing from zero, considering the accuracy of the methods. Potassium within cells, therefore, exchanges with potassium in serum to a very limited extent if at all under the conditions of these experiments. The same thing is shown in a different way by considering the transfer of potassium from serum into cells. Here there is evidence of slight chemical penetration (+7 per cent) in only

one of the five experiments. Radioactive penetration is also very slight in extent, but is greater than chemical penetration in three of the five experiments, suggesting that possibly a barely perceptible exchange of potassium ions has taken place. In no event is there anything approaching equilibrium.

Sodium—In Table II are summarized six experiments with sodium. The specific radioactivity of cells is apparently most irregular. However, the values in Experiments 1 and 5 should be excluded from consideration, since the amounts of sodium within

TABLE II

Exchange of Sodium between Cells and Serum

Experi- ment No.	Sodium added per liter whole blood	Specific radio- activity of added sodium	Concentration of sodium per liter water				Specific radio- activity of sodium in cells†	Transfers from serum to cells, per cent of amount added	
			Serum		Cells			Sodium, net	Radio- active sodium
			Initial	Final	Initial	Final			
	m.eq.	units* per m.eq.	m.eq.	m.eq.	m.eq.	m.eq.	units per m.eq.		
1	69	36	150	247	20	24	(−1.61)	+1	−7
2	70	181	152	241	12	61	1.31	+19	+13
3	131	50	152	315	28	97	0.97	+8	+9
4	130	50	148	320	16	76	2.45	−9	+19
5	78	62	146	253	24	20	(5.84)	+4	+14
6	75	62	151	242	11	36	1.52	+6	+7

The values in parentheses should be excluded from consideration, since the amounts of sodium within the cell at the end of the experiment are too small to permit even qualitative estimation of the ratio.

* Arbitrary units.

† The units are so defined in each experiment that the specific activity of the sodium in serum water is unity.

the cell at the end of the experiment (24 and 20 milliequivalents per liter of cell water) are too small to permit even qualitative estimation of the ratio. The cell figures for both radioactive and chemical concentrations within the cells are obtained as small differences between two large numbers. Neither is known with any great precision, and their ratio is even less precise. However, in Experiments 2, 3, 4, and possibly 6 the amounts present in the cells are sufficient to give some estimate of the specific radioactivity, which varies from 0.97 to 2.45. It is perhaps note-

worthy that the cells containing the largest amount of sodium (Experiment 3) gave the value most nearly approaching unity. The figures definitely suggest that equilibrium is established between sodium within cells and that in serum. Lack of technical accuracy makes it difficult to prove this point. Examination of the transfers of sodium indicates that under some circumstances appreciable amounts of sodium may enter red blood cells (Ex-

TABLE III

Exchanges of Phosphate between Cells and Serum

Experiment No.	Phosphorus added per 100 cc. whole blood	Specific radioactivity per mg. phosphorus	Temperature	Concentration of inorganic phosphorus per 100 cc. water				Specific radioactivity per mg. inorganic phosphorus in cells†	Transfers from serum to cells, per cent of amount added	
				Serum		Cells			Phosphorus, net	Radioactive phosphorus
				Initial	Final	Initial	Final			
	mg.	units*	°C.	mg.	mg.	mg.	mg.	units		
1	19	50	7	5.2	38.3	3.6	8.9	0.15	8	1
			38	5.3	30.9	6.8	23.2	1.80	26	44
2	5	300	7							
			38	4.7	11.2	3.9	6.8	(3.96)	7	41
3	101	31	7	4.2	181.0	0.6	17.2	2.44	5	11
			38	5.4	141.5	5.6	136.0	1.57	27	44
4	8	210	7	2.6	15.7	1.5	2.4	(0.96)	6	23
			38	3.3	16.7	2.9	9.3	1.04	26	27
5	24	55	7	4.7	47.6	0.4	−2.7	(0.00)	0	−4
			38	5.8	39.5	3.5	21.7	0.85	20	22
6	10	160	7	3.4	21.3	9.0	3.1	(1.88)	6	13
			38	4.1	17.0	3.8	14.0	1.42	30	39

The figures for cell specific radioactivity of four of the experiments at 7° (given in parentheses) are virtually meaningless.

* Arbitrary units.

† The units are so defined in each experiment that the specific activity of the inorganic phosphorus in serum water is unity.

periments 2, 3, and 4). From these experiments it is clear that sodium may sometimes cross the cell membrane and true equilibrium may exist.

Phosphorus—In Table III the data from six experiments with radioactive phosphorus are presented. Five of the six parallel experiments were carried out at 7° as well as 38°. In the experiments at 7° the same difficulty is encountered as in the sodium

experiments; that is, the amount of inorganic phosphate in the cells at the end of the experiment is too small to permit accurate determination of the specific radioactivity of cell phosphate. The figures for cell specific radioactivity of four of the five experiments at 7° are virtually meaningless. The remaining two (Experiments 1 and 3) give figures of 0.15 and 2.44 respectively. The figures at 38° in general are much more significant in view of the larger amounts of phosphate entering the cells; they are 1.80, 3.96, 1.57, 1.04, and 1.42 respectively. That is, in four of the five experiments radioactive phosphorus accumulated in cells out of proportion to the amount of inorganic phosphate present. This could only mean that at 38° phosphorus entered the cells freely, and that in some instances the radioactive phosphate was incorporated in some other form than inorganic phosphate.

No reliable information concerning the differences in behavior at 7° and at 38° can be obtained from the figures for the specific radioactivity, for the technical reasons which have just been mentioned. However, the differences clearly appear from the transfer figures. In every experiment there was virtually no chemical entry of inorganic phosphate at 7°, while in every instance there was marked entry at 38°. Radioactive entry was also trifling at 7° except in Experiment 4, while it was marked in all experiments at 38°.

<div align="center">DISCUSSION</div>

The results with potassium indicate that the potassium ions within the cell do not freely exchange with the potassium outside. This in general coincides with the experiments of Hahn, Hevesy, and Rebbe (5) with the rabbit. Since the potassium within and without the cells must exist largely as ionized potassium, the most reasonable explanation seems to be a membrane impermeability.

Danowski (2) has found that the normal serum potassium regularly decreases by a fraction of a milliequivalent when human blood to which no potassium has been added is incubated for 4 hours. Presumably such a transfer of potassium from serum to cells also took place in our experiments. If the small amount of potassium involved in this transfer had the same specific radioactivity as the rest of the potassium in serum, the increase in the

radioactivity of cell potassium would be barely detectable. In our experiments only a slight increase in cell radioactivity was noted. This fact is consistent with a unidirectional transfer of a small sample of potassium from serum to cells in Danowski's experiments. It clearly precludes the occurrence of extensive mixing of extracellular and cellular potassium, either by passive diffusion or by chemical equilibrium reactions.

The results with sodium are equivocal. Were the amounts of sodium present within the cell larger, some definite decision could be made. Certainly sodium may enter cells in some instances, so that some measure of permeability must exist.

Cohn and Cohn (1) have investigated the permeability of dog erythrocytes to radiosodium and they found considerable penetration. Because of the species difference their results are not directly comparable with ours.

The phosphate experiments represent a confirmation and an extension of the conclusions of Halpern (7) and of Hevesy and Aten (8). Inorganic phosphate is largely excluded from cells at 7° and enters actively at 38°, as indicated by both chemical and radioactive transfers. Furthermore, calculations of specific radioactivity show that at 38° radioactive phosphorus enters cells out of proportion to the amount of inorganic phosphate present. This demonstrates that some of the inorganic phosphate is rapidly converted into organic phosphorus compounds, within the cell. Both the total acid-soluble and the inorganic phosphorus have been found to be constant throughout the experiments, so the synthesis of the organic compounds must be balanced by the breakdown of other compounds. Active entry into the cells with continuous synthesis and breakdown of phosphorus compounds at 38° but not at 7° suggests an enzymatic process rather than simple diffusion.

SUMMARY

Radioactive salts of potassium, sodium, and phosphate were added *in vitro* to defibrinated human blood and incubated for 4 hours at 38°. Comparable experiments with phosphate at 7° were made.

No equilibrium developed between intracellular and extracellular potassium.

The amounts of sodium and the degree of radioactive penetration into the red blood cells were too small to show whether or not equilibrium existed, but some slight entry of sodium did take place.

Phosphate enters actively at 38°, but not at 7°. Active synthesis and breakdown of organic phosphorus compounds occurred within the cell at 38° but not at 7°. This was interpreted as evidence that the penetration of phosphates into the red cell is an active enzymatic process.

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CHEMICAL STUDIES ON POWDERED WOOL*

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The insolubility of keratins and their resistance to digestion by enzymes have yet to be adequately explained on the basis of protein structure. Many investigators have studied these properties and have attempted to alter them. Kühne (1) in 1877 observed that the keratin of hair was made digestible by pepsin when the surface area was increased by mechanical means. Powdered wool has been used by Harris and his coworkers in studies on the isoelectric point (2), the amino nitrogen content (3), and the effect of alkalies on keratins (4).

Other proteins have had their characteristic properties altered by being ground dry in a pebble mill (5, 6). Gelatin became soluble in cold water and delayed setting to a gel, while the swelling in acid solution of gliadin and glutenin was decreased after the material was ground (5). Boissevain (6) found that when gelatin was ground for longer periods it was not only soluble in cold water but did not set to a gel even in an ice box. He also observed that water-soluble protein, peptone, and polysaccharide components were produced when tubercle bacilli were ground with small steel balls in a Pyrex bottle.

In recent preliminary studies (7), it was observed that after wool was ground the powdered material was digested by both trypsin and pepsin. An appreciable fraction of the nitrogen and sulfur of the powdered keratin was soluble in water. That the powdered wool was contaminated with a large amount of inorganic material from the ball mill (porcelain-lined and charged with quartz pebbles) was shown by the high content of ash (48 per cent) in a batch ground 125 hours.

This paper deals with more complete chemical studies on powdered wool prepared so as to minimize contamination.

EXPERIMENTAL

To insure an adequate supply of uniform starting material a large quantity of wool was carefully cleaned and defatted. It contained 16.02 per cent nitrogen, 3.77 per cent sulfur, 11.83 per cent cystine, and 0.75 per cent ash; the amino nitrogen was 1.91 per cent of the total nitrogen.

A steel ball mill, approximately 7 liters in capacity, was constructed. The balls were of tool steel, each weighing about 66 gm. The mill was equipped with a revolution counter and when charged with 100 balls and a batch of wool (150 gm.) ran at a speed of 40 to 41 revolutions per minute.

When the defatted wool was merely clipped into short fibers with scissors, the fibers formed into small felt pads which resisted the grinding in the ball mill. The tendency to form pads was prevented by first cutting the wool into very fine pieces in a Wiley mill. Subsequent grinding of the material in the ball mill was continued for varying lengths of time. To separate the ground material into fractions of different particle size it was placed in the top of a graded series of sieves (100 to 325 mesh) and shaken for several hours in a Ro-Tap machine. The separation was considered complete when only traces of the powder passed from one sieve into another.

The fractions of the powdered wool of different particle size were then extracted with water. The samples (2.5 gm.) were suspended in water (30 ml.) by mechanical stirring and the suspension was centrifuged. The supernatant liquid was removed and the process repeated three times. The residue after extraction was dried in a vacuum oven.

The total nitrogen of the powdered wool, the water extract, and the residue was determined by the Kjeldahl method and amino nitrogen by the Kendrick and Hanke modification (8) of the Van Slyke method. The latter method was further modified according to the suggestions of Kanagy and Harris (3), to allow a more accurate measure of the low values obtained on solid proteins; a macro reaction chamber was combined with a microburette and a small volume (15 ml.) of nitrous acid was used. The total

sulfur of the powdered material was determined by the Parr bomb and of the water-soluble material by the Benedict-Denis method. Cystine was determined by the Shinohara method (9) on hydrolysates both of the powdered material and of the residue obtained on evaporation of the water-soluble material. The values for the intermediate oxidation products of cystine were obtained by the method outlined by Lavine (10). The inorganic sulfate content of the water-soluble fractions was determined gravimetrically after precipitation with barium chloride.

TABLE I

Effect of Grinding on Cystine Content of Wool

Batch No.	Particle size	Ball mill revolutions	Ash	Cystine*		
				Unextracted material (a)	Water-soluble fraction	Residue after extraction
	mesh		*per cent*	*per cent*	*per cent of (a)*	*per cent*
WM-1	325	75,000	1.47	11.77	0.89	12.06
WM-2	100–150	100,000	1.01	11.21	0.83	11.13
"	150–200	100,000	1.11	11.27	0.87	11.30
"	200–250	100,000	1.12	10.94	0.95	11.03
"	250–325	100,000	1.01	11.15	0.93	11.09
"	325	100,000	1.05	10.89	0.91	11.16
WM-5	325	200,000	0.98	10.52	1.12	10.64
C-6	200–270	240,000	1.50	11.00	1.11	11.27
WM-6	325	300,000	1.04	9.97	2.01	10.36
WM-7	325	400,000	0.97	9.27	2.37	9.98
WM-8	325	500,000	0.97	9.03	2.47	9.48
WM-9	325	1,000,000	1.13	8.42	5.60	9.03

* All values were calculated on an ash-free basis. The untreated wool contained 11.83 per cent cystine and 0.75 per cent ash.

The samples of powdered material were always dried in a vacuum oven at 65° and stored in a vacuum desiccator over phosphorus pentoxide. At least duplicate analyses were made in every case.

Analysis of the fractions of varying particle size showed that the composition of the powdered wool did not vary with the size of the particles. The results from a typical batch (Batch WM-2) are included in Tables I to III. On the other hand the time of grinding apparently produced marked changes in the composition.

The results recorded were for the most part obtained on material that passed the 325 mesh sieve (325 openings per linear inch); however, the fractions of other particle size in each batch were analyzed and showed similar changes throughout. The last batch in Tables I to III (Batch WM-9) was ground continuously for over 2 weeks; there was no noticeable increase in the temperature of the ball mill during this period. The low ash content

TABLE II

Effect of Grinding on Nitrogen Content of Wool

Batch No.	Particle size	Ball mill revolutions	Nitrogen*					
			Unextracted material		Water-soluble fraction		Residue after extraction	
			Total (a)	Amino	Total	Amino	Total (b)	Amino
	mesh		*per cent*	*per cent of (a)*	*per cent of (a)*	*per cent of (a)*	*per cent*	*per cent of (b)*
WM-1	325	75,000	15.79	1.78	1.17	0.39	15.84	1.53
WM-2	100–150	100,000	15.63	1.98	1.28	0.39	16.07	1.70
"	150–200	100,000	15.78	1.97	1.21	0.39	16.45	1.60
"	200–250	100,000	15.39	2.03	1.19	0.43	16.08	1.70
"	250–325	100,000	15.61	1.95	1.22	0.40	16.34	1.58
"	325	100,000	15.57	2.03	1.62	0.46	16.31	1.61
WM-5	325	200,000	15.81	2.13	2.64	0.41	16.27	1.83
C-6	200–270	240,000	15.35	2.02	3.38	0.41	15.74	1.73
WM-6	325	300,000	15.46	2.08	4.95	0.40	15.99	1.78
WM-7	325	400,000	15.73	2.03	5.70	0.48	15.79	1.76
WM-8	325	500,000	15.70	2.23	6.49	0.47	15.58	1.93
WM-9	325	1,000,000	16.31	2.13	11.12	0.44	16.29	1.80

* All values were calculated on an ash-free basis. The total nitrogen content of the untreated wool was 16.02 per cent; the amino nitrogen was 1.91 per cent of the total.

of the ground material (Table I) indicated much less contamination than in the powdered wool prepared in the preliminary work (7).

The greatest changes were observed in the cystine values (Table I). The water-soluble fraction before hydrolysis gave only faintly positive color tests for cystine; after hydrolysis appreciable amounts of cystine were present. The residue after extraction gave cystine values slightly larger than those of the unextracted material, but this increase may have been relative rather than absolute.

The nitrogen content of the powdered keratin (Table II) was little affected by the grinding process although the residue after water extraction contained slightly more nitrogen than the unextracted material. The nitrogen content of the water-soluble fraction increased as the time of grinding was prolonged. However, the amino nitrogen of the extract varied little, which was unexpected in view of the increase in its cystine content. The total amino nitrogen content of the water-soluble extract and the

TABLE III

Effect of Grinding on Sulfur Content of Wool

Batch No.	Particle size	Ball mill revolutions	Unextracted material (a)	Sulfur*					Residue after extraction
				Water-soluble fraction					
				Total	Inorganic sulfate	"Sulfinic acid"	Cystine		
	mesh		*per cent*	*per cent of (a)*	*per cent of (a)*	*per cent of (a)*	*per cent of (a)*		*per cent*
WM-1	325	75,000	3.82	2.31	1.43	0.68	0.60		3.82
WM-2	100–150	100,000	3.84	4.43	3.07	0.66	0.65		3.50
"	150–200	100,000	3.82	4.06	3.01	0.79	0.68		3.63
"	200–250	100,000	3.75	4.18	3.25	0.70	0.74		3.59
"	250–325	100,000	3.68	4.07	3.02	0.68	0.75		3.65
"	325	100,000	3.69	4.40	3.45	0.69	0.72		3.70
WM-5	325	200,000	3.63	6.06	4.24	1.16	0.87		3.58
C-6	200–270	240,000	3.81	4.96	2.59	0.66	0.98		3.86
WM-6	325	300,000	3.71	8.16	5.66	1.09	1.44		3.62
WM-7	325	400,000	3.74	8.31	5.28	1.05	1.56		3.54
WM-8	325	500,000	3.55	8.69	5.29	1.11	1.67		3.52
WM-9	325	1,000,000	3.70	12.10	6.17	1.48	3.39		3.52

* All values were calculated on an ash-free basis. The sulfur content of the untreated wool was 3.77 per cent.

residue accounted for 105 to 111 per cent of the amino nitrogen of an equivalent quantity of the unextracted material.

The sulfur content of the powdered wool and of the residue after extraction was affected only slightly by the grinding process (Table III). On the other hand the water-soluble material contained increasing amounts of sulfur, as it did of nitrogen, with prolonged grinding.

The sulfur partition of the water-soluble fraction was of considerable interest. The inorganic sulfates in every case accounted

for at least 50 per cent of the total soluble sulfur. The method used for the determination of intermediate oxidation products of cystine (10) measures sulfinic acid, cystine disulfoxide, and sulfenic acid. The latter compounds are not as stable as sulfinic acid and are more rapidly changed by oxidation. Whether the intermediate oxidation products of cystine were calculated as sulfinic acid or as the disulfoxide, a slight increase in this fraction occurred as the grinding was prolonged. When calculated as sulfinic acid, the sulfur fractions accounted for 85 to 117 per cent of the water-soluble sulfur, while when calculated as the disulfoxide, they accounted for 90 to 127 per cent.

<div align="center">DISCUSSION</div>

The results obtained in this investigation suggest that grinding in a ball mill produces a degradation of wool protein accompanied by oxidative changes. Thus, in the early stages of cleavage, some of the protein would be split into smaller units, the cystine perhaps being oxidized to the disulfoxide. If the cystine was split to cysteine, it would first be oxidized to sulfenic and then to sulfinic acid. It has been shown by Lavine (10) that the disulfoxide undergoes dismutation to form cystine, sulfenic, sulfinic, and cysteic acids. The final stage in the oxidation of the cystine would be through cysteic acid to inorganic sulfates. The fact that over half of the water-soluble sulfur is in the form of sulfates shows that considerable oxidation takes place.

A comparison of powdered wool prepared from fibers longer than those produced by the Wiley mill confirms the suggestion of oxidation. A typical batch of such wool (Batch C-6) had less water-soluble sulfur and inorganic sulfates than a lot (Batch WM-5) which was cut in the Wiley mill and ground for an even shorter period. Pad formation apparently not only protected a portion of the wool from being ground, but also partially prevented oxidation. In the porcelain ball mill with quartz pebbles (7) 60 per cent of the water-soluble sulfur was in the form of cystine; in the steel ball mill only 14 to 28 per cent was in this form. Perhaps the metal catalyzed oxidation of the sulfur-containing compounds.

From studies on the dry grinding of gelatin and tubercle bacilli, Boissevain (6) concluded that the process was similar to acid hydrolysis and consisted of breaking large molecules into smaller

units.   Hydrolytic action alone cannot explain the results obtained in the present investigation.   The problem is further complicated by the suggestion of several workers that wool keratin is not an individual protein and that the medulla and cortex probably differ in composition.   The mechanical process may produce a water-soluble fraction merely by bringing the soluble material into contact with the solvent.   On the other hand a mechanical degradation similar to hydrolysis may be required to convert insoluble keratin into soluble protein.   Whatever the process of degradation, it is accompanied by oxidative changes.

It is proposed to alter the mill so that the wool can be ground in an inert atmosphere.   Such a study should help to determine the rôle of oxygen in the degradation process.

SUMMARY

1. The prolonged grinding of wool in a steel ball mill produced little change in the total nitrogen and sulfur content. The cystine content of the powdered material decreased appreciably. Aqueous extracts contained increased amounts of nitrogen, amino nitrogen, cystine, inorganic sulfates, and the intermediate oxidation products of cystine.   The sulfur compounds accounted for 85 to 117 per cent of the total water-soluble sulfur.

2. The production of powdered wool was accompanied by oxidation, since the cystine sulfur accounted for a decreased percentage of the total sulfur and more than 50 per cent of the water-soluble sulfur was in the form of inorganic sulfates.

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KETOGENIC ACTION OF SHORT CHAIN, EVEN NUMBERED CARBON FATTY ACIDS IN CARBOHYDRATE-FED ANIMALS

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All methods for the determination of acetone bodies in the blood give a value of 0.2 to 2.0 mg. per cent of total acetone in the blood of normal fed rats, dogs, rabbits, and humans (1). It is not known whether this small value is due to the presence of acetone bodies or the presence of some interfering material. When this value is measureably increased, we say there is a ketosis. Such an increase in the level of acetone bodies in the blood has been assumed to be dependent upon a lack of oxidizable carbohydrate such as exists during fasting and in phlorhizin or pancreatic diabetes. Heretofore such a ketosis has not been believed to occur unless the liver glycogen has been greatly depleted. Under normal circumstances fasting has been assumed to be essential. Data are presented here which show that short chain, even numbered carbon fatty acids are ketogenic and produce a ketosis as measured by the acetone body level in the blood when fed to normal carbohydrate-fed rats with large amounts of glycogen in their livers.

EXPERIMENTAL

Young adult albino rats were removed from the stock diet and for 4 hours before being fed the fatty acid esters and thereafter until sacrificed were allowed access to water and cubes of sucrose *ad libitum*. In Experiment 4 they were removed from the stock diet 12 hours earlier and sucrose allowed so that the intestinal contents would be reduced and permit observations on absorption rate. Male rats were used in Experiments 1 to 3 and female rats in Experiment 4. The latter averaged 169 gm. in weight, while the male groups averaged from 180 to 195 gm. There were three

TABLE I

Influence of Feeding Fatty Acid Esters upon Blood Acetone Bodies of Carbohydrate-Fed Rats

Time after feeding

Experiment No.	Ethyl ester fed	Dose per sq. dm. body surface	2 hrs. Liver glycogen	2 hrs. Blood acetone bodies	3 hrs. Liver glycogen	3 hrs. Blood acetone bodies	4 hrs. Liver glycogen	4 hrs. Blood acetone bodies	6 hrs. Liver glycogen	6 hrs. Blood acetone bodies	8 hrs. Liver glycogen	8 hrs. Blood acetone bodies
		m.u	per cent	mg. per cent	per cent	mg. per cent	per cent	mg. per cent	per cent	mg. per cent	per cent	mg. per cent
1	Ethyl alcohol	2.70					5.41	0.46				
	C_4, butyrate	2.70					5.53	2.50				
	C_6, caproate	1.80					4.90	4.06				
	C_8, caprylate	1.35					3.40	4.00				
	C_{12}, laurate	0.90					4.54	0.33				
	C_{18}, oleate	0.60					4.01	0.50				
2	Ethyl alcohol	2.70	5.96	0.90			5.50	1.05	6.57	0.17	8.97	0.30
	C_4, butyrate	2.70	9.33	2.86	6.80	5.66	4.41	2.10	7.41	3.17	11.60	1.51
	C_6, caproate	1.80	8.48	7.68	5.56	6.36	7.97	6.58	5.25	5.38	11.93	3.40
	C_{12}, laurate	0.90	8.13	0.80	6.01	0.74	9.49	0.33	9.30	0.15	10.08	0.61
	C_{18}, oleate	0.60	6.42	0.59	6.96	0.65	4.10	0.35	6.59	0.21	12.05	0.21
3	Ethyl alcohol	1.35	10.30	0.70	2.93	1.40	5.46	1.34	4.82	0.99	2.68	1.08
	C_{10}, caprate	1.08	3.43	3.90	3.42	3.96	3.22	2.91	4.87	0.76	2.65	1.92
	C_{14}, myristate	0.78	5.86	0.58	3.23	1.00	4.46	0.41	4.24	1.07	2.55	0.47
	C_{16}, palmitate	0.67	4.93	1.11	2.27	1.05	3.99	0.99				
4	Ethyl alcohol	3.00	8.45	1.36	7.44	1.03	9.54	1.08			7.25	0.69
	C_8, caprylate	3.00	9.24	5.66	18.53	11.96	7.28	13.76			5.10	17.20
	C_{10}, caprate	2.40	6.57	6.26	9.33	8.66	6.73	8.91			7.21	9.21
	C_{12}, laurate	2.00	10.58	0.95	9.41	0.89	10.20	0.93			5.15	1.34
	C_{14}, myristate	1.71	8.56	0.42	7.94	0.64	9.51	0.64			6.32	0.99

rats in each group and all figures represent the average of the determinations made on the members of the group.

The fatty acid esters which were fed by stomach tube came from the Eastman Kodak Company with the exception of the ethyl oleate which we prepared ourselves.   This ester was used in place of the stearate, because the latter is not liquid at body temperature. Acetic acid could not be fed, for the free acid and even more so the ethyl ester are too toxic in the doses necessary to produce a definite rise in the blood acetone bodies.   The other fatty acids were fed as the ethyl esters, because these are so much less toxic than the free acids.   The control rats were given ethyl alcohol equivalent to the dose of butyrate in Experiments 1 and 2, of caprate in Experiment 3, and of caprylate in Experiment 4.   In

TABLE II

Absorption Rate of Ethyl Esters in Experiment 4

Ester fed	Per cent absorbed at end of period			
	2 hrs.	3 hrs.	4 hrs.	8 hrs.
Caprylate...............	39	44	65	95
Caprate.................	32	54	56	69
Laurate.........................	23	33	44	70
Myristate.......................	30	47	74	85

each case the rats which were fed the fatty acids higher than these were also fed quantities of ethyl alcohol such that an equal amount of the ethyl radical was fed to all of the rats in a given experiment. The doses of fatty acid esters were based on the assumption that all of the carbon atoms in the fatty acids may be converted to acetone bodies.

At the end of the various periods after feeding, the rats were anesthetized with sodium pentabarbital solution, administered intraperitoneally, and an oxalated blood specimen taken from the severed abdominal aorta.   Acetone bodies were determined on these specimens by the method of Barnes and Wick (1).   All of the results are expressed in terms of acetone.   Glycogen determinations were carried out on the livers according to the method of Good, Kramer, and Somogyi (2).

Results

Group averages are presented in Table I. The data show very clearly that fatty acids with an even number of carbon atoms, from C_4 to C_{10}, may be ketogenic in the carbohydrate-fed organism with adequate liver glycogen supplies. The fatty acids from C_{12} and up are not ketogenic under the same conditions. That these results are not primarily due to differences in the rates of absorption may be seen from Table II. These data were obtained in Experiment 4 with the same method of measuring absorption that we have applied to fats (3).

DISCUSSION

The most likely explanation of the ketogenic activity of short chain (C_{10} and lower), even numbered carbon fatty acids in the presence of an excess of carbohydrate appears to us to be associated with the inability of the body to store fat containing short chain fatty acids. Under ordinary dietary conditions the body fat contains practically no fatty acid lower than C_{14} although C_{12} may be stored when present in the diet in significant amounts (4, 5). There is no appreciable storage of capric acid (C_{10}) when it is present in the diet in reasonable quantities (5) and only 15 per cent of the carcass fat is made up of this fatty acid when 50 per cent of the calories in the diet is in the form of tricaprin (6). Caprylic (C_8) and lower acids cannot be stored at all (4, 5, 7). Although lower fatty acids may contribute to the synthesis of longer chain acids and there is evidence (7-10) that they are able to change the nature of the fatty acids in deposit fat, we believe that they are in large part converted to acetone bodies and utilized at once, because the organism is unable to store them as such. Deuel *et al.* (11) have offered a similar explanation for the failure of a fat composed of long chain fatty acids such as cottonseed oil to give rise to glycogen, while tributyrin in equivalent amounts causes an appreciable deposition of hepatic glycogen. They suggested that since butyric acid cannot be stored in the body it is utilized and the glycerol of the tributyrin is free to be converted to glycogen. The glycerol esters of oleic, palmitic, and stearic acids of which cottonseed oil is chiefly composed on the other hand are normal components of storage fat of rats, can be

deposited as such, and the glycerol is not free to be converted to glycogen.

That the ketogenic activity of short chain fatty acids in the presence of carbohydrate is not more evident in normal blood acetone values is probably due to the very small amount of these lower fatty acids in the ordinary diet of man and animals.

The observations here reported raise the possibility that the "normal" blood acetone body levels found with all methods (1) and which persist even on a diet of pure carbohydrate are actually due to acetone bodies and result from protein catabolism. The ketogenic amino acids may be oxidized in a similar manner to their lower fatty acid counterparts.

SUMMARY

Capric acid and other fatty acids with an even number of carbon atoms and with shorter chains are ketogenic even when adequate amounts of carbohydrate are fed. The blood acetone body level is significantly raised when the ethyl esters of these fatty acids (C_4 to C_{10}) are fed to rats with liver glycogen levels of 4 per cent and higher. Fatty acids of C_{12} to C_{18} are not ketogenic.

It is suggested that the lower fatty acids are ketogenic in the presence of large amounts of liver glycogen, because unlike the longer chain acids they cannot be stored as body fat and hence are oxidized at once.

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THE ORIGIN AND COMPOSITION OF THE HEMICELLU-LOSES OBTAINED FROM HARDWOODS*

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Norman (1) has reviewed some of the recent work on the hemicelluloses. Spoehr and Milner (2), Niemann, Roberts, and Link (3), and Campbell (4) have described the isolation of starch from woody tissues. Campbell (4) states that wood starch is an acid polysaccharide, approximately 90 per cent of which is composed of anhydroglucose residues. He suggests that the remainder consists of an aldobionic acid anhydride. Buston (5) suggests that the hemicelluloses of lignified cell walls are derived from hexose polysaccharides. O'Dwyer (6) isolated from the sap-wood of the English oak a Hemicellulose A which gave a deep blue color with iodine solution. She states that the blue color reaction given by this hemicellulose with iodine is due to anhydroglucose units in the molecular structure. She also states that her work supports the theory of Buston and Campbell as to the origin of hemicelluloses in wood.

The present investigation was undertaken to determine whether hemicelluloses obtained from various hardwoods, and from the heart-wood and sap-wood of the same tree, are the same or different. The hardwoods investigated were white birch wood, *Betula*

populifolia, Marshall, lemon wood, *Citrus limonia*, Osbeck, and heart-wood and sap-wood of black locust, *Robinia pseudacacia*, L.

EXPERIMENTAL

Extraction of Hemicelluloses—The hemicelluloses were isolated as described by Anderson (7) and by Anderson, Seigle, Krznarich, Richards, and Marteny (8). The yields of the crude hemicelluloses before purification averaged approximately 14 per cent of the wood used.

Fractionation of Hemicelluloses—In fractionating the hemicelluloses, the alkaline extracts were acidified with hydrochloric acid to an optimum pH and the less soluble portion was centrifuged from the solution. The more soluble material was then precipitated by addition of ethanol. The hemicelluloses were thus separated into Fractions A and B before chlorination of the wood and Fractions C and D after chlorination of the wood. Fractions A and C contain the less soluble portions of the hemicelluloses, while Fractions B and D contain the more soluble portions. The separation of the hemicelluloses by this method is not sharp. However, the analytical results prove that the two fractions differ in composition and properties. The less soluble portions contain the smaller per cent of uronic acid and probably have the larger molecules.

Removal of Starch from Hemicelluloses—Starch was isolated from the hot water extract of lemon wood and the sap-wood of black locust by the freezing method of Spoehr and Milner (2). All of the hemicelluloses obtained from these two woods before chlorination of the wood gave blue colorations with iodine. The hemicelluloses obtained from these two woods after chlorination of the wood gave no color with iodine. No starch was isolated from the hot water extract of the heart-wood of black locust nor from white birch wood. The hemicelluloses obtained from these two woods gave no color with iodine.

O'Dwyer (6) has described the action of taka-diastase and of dilute acids on hemicelluloses which gave a blue color with iodine. In the present investigation repeated attempts were made to remove from the hemicelluloses the material which gave blue color with iodine. For this purpose taka-diastase, malt diastase, and even a 2 per cent solution of hydrochloric acid were tried sepa-

rately on small amounts of the hemicelluloses. The hemicelluloses which were treated with taka-diastase showed a decrease in the depth of the blue coloration and some of them eventually gave with iodine the purple color characteristic of the larger dextrin molecules. However, when a hemicellulose was used that originally gave a blue coloration with iodine, taka-diastase did not remove from it all the material that gave this color unless the treatment was prolonged for more than a week.

The following general procedure was used on all the hemicelluloses that originally gave a coloration with iodine. The hemicelluloses were dissolved in a 4 per cent solution of sodium hydroxide and the solutions were filtered. The filtrates were made faintly acid with hydrochloric acid and 1 gm. of taka-diastase was added to each 3 liters of solution. The solutions were allowed to stand at a temperature of 35° for 24 hours. This treatment was repeated a second time. The hemicelluloses were then precipitated by ethanol and isolated as already described. In no case did this treatment remove all the material that gave the blue coloration with iodine.

The hemicelluloses that originally gave no coloration with iodine were not treated with taka-diastase.

Purification of Hemicelluloses—In order to purify the hemicelluloses, they were dissolved in 4 per cent solutions of sodium hydroxide and the solutions were filtered. The filtrates were made faintly acid with hydrochloric acid and liquid bromine was added until, after shaking, free bromine remained in the solution for 24 hours. 3 volumes of ethanol were then added to precipitate the hemicelluloses and dissolve the colored material. The purified hemicelluloses were centrifuged from the solutions, washed with 85 per cent ethanol until free of halides, and dried. This procedure was repeated until the dry hemicelluloses were white. The fractions were separated by variation in the amount of ethanol added into less soluble and more soluble portions, Fractions A_1 and A_2, B_1 and B_2, C_1 and C_2, and D_1 and D_2.

Methods of Analysis—Anderson, Seigle, Krznarich, Richards, and Marteny (8) give references to the literature dealing with the methods of analysis. They are also described in the texts by Dorée (9) and van der Haar (10). Specific rotations were made

by dissolving the hemicelluloses in a 2 per cent solution of sodium hydroxide. In the xylan determinations the weights of furfural phloroglucide were corrected for the furfural given by the uronic acid.

The presence of an ether-linked methoxyl group in all the hemicelluloses was established by the method of von Fellenberg (11) and Denigès (12). The presence of a uronic acid was established by the naphthoresorcinol test ((10) pp. 55–57) and by the yield of carbon dioxide by the method of Lefèvre and Tollens (13).

Hydrolysis of Hemicelluloses—Weighed amounts of the various hemicelluloses were mixed with 10 times their weights of a 4 per cent solution of sulfuric acid and the mixtures were heated in baths of boiling water for 15 hours. The cold solutions were neutralized by barium hydroxide and barium carbonate and treated with decolorizing carbon. The filtrates were concentrated under reduced pressure to syrups. The barium salts of the aldobionic acids were precipitated by addition of ethanol and purified by repeated solution in water and reprecipitation by ethanol. The sugars were isolated from the syrups by crystallization from glacial acetic acid and ethanol.

Two barium salts were obtained by hydrolysis of the hemicellulose from lemon wood. One of these showed $[\alpha]_D^{25} = +65.16°$ and gave 8.21 per cent carbon dioxide, by the method of Lefèvre and Tollens (13), and 5.9 per cent methoxyl. The other showed $[\alpha]_D^{25} = +75°$ and gave 10.82 per cent carbon dioxide. The barium salt of a monomethoxyuronic acid combined with two xylan groups should give 8.15 per cent carbon dioxide and 5.7 per cent methoxyl. The barium salt of a monomethoxyuronic acid combined with one xylan group should give 10.79 per cent carbon dioxide. Evidently the first of these salts contains two xylan groups combined with the uronic acid, while the second salt contains one xylan group.

A calcium salt obtained by hydrolysis of the hemicellulose from black locust sap-wood showed $[\alpha]_D^{25} = +70°$, and gave 12.2 per cent carbon dioxide. The calcium salt of a monomethoxyuronic acid with one xylan group should give 12.25 per cent carbon dioxide.

Analysis of the barium salts obtained by hydrolysis of the hemicelluloses from the heart-wood of black locust and white birch

wood showed that they were mixtures of the salts of a mono-methoxyuronic acid combined with one xylan group and with two xylan groups. Their rotations and analyses were intermediate between the two salts obtained from lemon wood.

Large amounts of crystalline d-xylose were obtained by hydrolysis of the hemicelluloses from all four of the woods. This was identified by its $[\alpha]_D^{25} = +18.3°$ and by conversion to the boat-shaped crystals of cadmium bromide-cadmium xylonate. No other pentose sugar could be detected. When hemicelluloses that gave no coloration with iodine were hydrolyzed, all but a trace of the sugar could be obtained as crystalline d-xylose. This indicated that no other sugar was present.

Small amounts of crystalline d-glucose were obtained by hydrolysis of the hemicelluloses from the sap-wood of black locust and from lemon wood before chlorination. This was identified by its $[\alpha]_D^{25} = +52.5°$ and by conversion to potassium acid saccharate.

Coloration of Hemicelluloses by Iodine Solution—The blue coloration shown by some hemicelluloses when treated with iodine solution may be due to the admixture of starch with the hemicelluloses or, as suggested by O'Dwyer (6), to the presence of anhydroglucose units in the hemicellulose molecule. The following observations seem to support the suggestion of O'Dwyer.

Small amounts of purified hemicelluloses which gave a blue coloration with iodine were dissolved in a 2 per cent solution of sodium hydroxide and the solutions were then made faintly acid with hydrochloric acid. Filtered saliva and taka-diastase were added separately to portions of the above solutions. After standing for 24 hours these solutions still gave a blue coloration with iodine. For comparison with the above experiment small amounts of a hemicellulose which gave no color with iodine were mixed with corn-starch. This mixture was treated with sodium hydroxide and dilute hydrochloric acid, as described above. It gave a blue coloration with iodine. Filtered saliva and taka-diastase were added separately to portions of the resulting solutions. After standing for a few hours these solutions gave no coloration with iodine. Evidently within 5 hours both taka-diastase and filtered saliva will hydrolyze completely starch that has been mixed

with the hemicellulose. On the other hand, neither of these enzymes will hydrolyze completely within 5 hours the material that gives the blue color to a hemicellulose obtained from wood that contains starch.

This fact indicates that the material which gives a blue coloration to some hemicelluloses when treated with iodine is a part of the hemicellulose molecule. Since d-glucose was repeatedly obtained by hydrolysis of hemicelluloses that were colored blue by iodine but never from those that gave no coloration with iodine, it seems probable that anhydroglucose groups in the hemicellulose molecule are the cause of the blue coloration.

Analyses of Hemicelluloses—During the course of the investigation many lots of the hemicelluloses were analyzed. The results vary widely, depending on the exact procedure followed in purifying the material. These hemicelluloses are mixtures of varying molecular size. The larger molecules are less soluble than the smaller molecules and probably approximate the composition of the original hemicelluloses. The smaller molecules probably result in part from partial hydrolysis of the original hemicelluloses. However, the original hemicelluloses in the wood apparently vary in molecular size.

In Table I are recorded typical analytical results for the least soluble portion, Fraction A_1, and the most soluble portion, Fraction B_2, of the hemicelluloses obtained before chlorination of the wood, and the least soluble portion, Fraction C_1, and most soluble portion, Fraction D_2, of the hemicelluloses obtained after chlorination of the wood. Fractions A_2, B_1, C_2, and D_1, intermediate in solubility between those listed in Table I, also gave analytical results which were intermediate between those given in the table.

Discussion of Analytical Results—The analyses given in Table I of Fractions A_1 and C_1, the least soluble fractions, vary considerably. This is also true of Fractions B_2 and D_2, the most soluble fractions of the hemicelluloses. The chief causes of variation in the analyses of corresponding fractions of the different hemicelluloses are (1) incomplete separation of hemicelluloses of different molecular size, (2) the presence of hemicelluloses containing anhydroglucose units, (3) the presence of traces of starch and pectic materials. The first two of these factors influence greatly the analytical results. Hemicelluloses of small molecular size

TABLE I

Analyses of Hemicelluloses Obtained from Various Hardwoods

	Fraction A_1	Fraction B_2	Fraction C_1	Fraction D_2
Black locust sap-wood				
Carbon dioxide, %	1.69	2.56	1.65	2.25
Methylated uronic acid, %	7.92	12.10	7.80	10.63
Xylan, %	90.50	76.54	93.00	91.00
Total %	98.42	88.64	100.80	101.63
Methoxyl, %	1.39	1.83	1.23	1.58
$[\alpha]_D^{25}$, *degrees*	−84.62	−45	−84.90	−76
Equivalent weight	2603	1719	2666	1955
Xylan units	18.1	10.0	18.6	13.2
Black locust heart-wood				
Carbon dioxide, %	1.68	2.50	1.85	2.50
Methylated uronic acid, %	7.94	11.8	8.74	11.80
Xylan, %	91.70	89.00	91.10	87.00
Total %	99.64	100.80	99.84	98.80
Methoxyl, %	1.18	1.75	1.25	1.75
$[\alpha]_D^{25}$, *degrees*	−78.24	−58.16	−83.73	−60.00
Equivalent weight	2619	1760	2378	1760
Xylan units	18.2	11.7	16.4	11.7
Lemon wood				
Carbon dioxide, %	2.01	3.37	1.89	3.11
Methylated uronic acid, %	9.50	15.93	8.93	14.70
Xylan, %	90.39	84.02	90.56	85.46
Total %	99.89	99.95	99.49	100.16
Methoxyl, %	1.43	2.38	1.20	1.75
$[\alpha]_D^{25}$, *degrees*	−86.1	−61.00	−86.0	−76
Equivalent weight	2189	1306	2328	1414
Xylan units	15	8.3	16	9.1
White birch wood				
Carbon dioxide, %	1.83	2.35	1.68	2.06
Methylated uronic acid, %	8.65	11.11	7.95	9.74
Xylan, %	92.19	89.10	93.00	90.78
Total %	100.84	100.21	100.95	100.52
Methoxyl, %	1.50			
$[\alpha]_D^{25}$, *degrees*	−86.5	−74.4	−90.0	−75.17
Equivalent weight	2404	1872	2624	2133
Xylan units	16.6	12.6	18.1	15

have a higher per cent of methylated uronic acid and a lower per cent of xylan. They are less strongly levorotatory. Their presence in Fraction A_1 will cause an increase in the percentage of methylated uronic acid and a decrease in the percentage of xylan, the equivalent weight, the $[\alpha]_D^{25}$, and in the number of xylan units. It is very difficult to purify to the same extent corresponding fractions of the different hemicelluloses.

The hemicelluloses from two of the woods apparently contain transition hemicelluloses intermediate between starch or dextrin and the regular hemicelluloses. These intermediate products contain the methylated uronic acid and the xylan chain but have also some anhydroglucose units in the xylan chain. These transition hemicelluloses apparently are less strongly levorotatory than the regular hemicelluloses. They cause a decrease in the percentage of xylan and $[\alpha]_D^{25}$.

The purified hemicelluloses which give no coloration with iodine solution consist of a monomethylated uronic acid which is joined by a glycosidic union to a series of xylan groups. The analyses in Table I indicate that the least soluble hemicelluloses contain from 17 to 19 xylan units per molecule of uronic acid. Probably the larger of these numbers is the more nearly correct, since it is difficult to remove completely all of the smaller hemicellulose molecules.

The analyses indicate that the smaller hemicelluloses usually contain between ten and twelve xylan groups. However, they vary in xylan content from approximately 8 to 15 units.

Origin of Hemicelluloses—It appears that a mixture of hemicelluloses is present in the hardwoods investigated. Each contains 1 molecule of a monomethylated uronic acid joined by glycosidic union to a series of *d*-xylan groups. This type of mixture appears to occur in the heart-wood and the deeper cell wall layers of the sap-wood. A third type of hemicellulose apparently has the same general structure as just described except that it has some anhydroglucose units in the molecule.

It is significant that hemicelluloses which are colored blue or pink by iodine solution are not obtained from the heart-wood nor from any of the woods after chlorination. They are obtained from woods that give a blue coloration with iodine. These facts suggest that the hemicelluloses are formed from starch or dextrin.

It is conceivable that as the starch is laid down inside the cell it is gradually transformed into hemicelluloses. The deeper layers of hemicellulose in the cell wall might be composed of hemicelluloses with no anhydroglucose molecules, while the hemicellulose layers next the inner surface of the cell wall might be in a transition stage between starch or dextrin and hemicellulose. These latter hemicelluloses would be dissolved out first by treatment with the alkaline solution. Chlorination of the wood would then remove lignin and other encrusting materials and expose the deeper layers of mature hemicellulose to the action of the alkaline solution.

Apparently the hemicelluloses are attached to some substance in the cell wall, probably through their carboxyl group. This is indicated by the fact that, while they cannot be dissolved out of the wood by hot water, they are soluble to some extent in water, once they have been dissolved in sodium hydroxide.

SUMMARY

Hemicelluloses have been prepared from white birch wood, lemon wood, and the sap-wood and heart-wood of black locust before and after chlorination of the wood. Lemon wood and the sap-wood of black locust contained starch. The other two woods were starch-free. All of the hemicelluloses obtained from lemon wood and the sap-wood of black locust before chlorination gave a blue or pink coloration with iodine solution. Analyses of these hemicelluloses show that those which are not colored by iodine solution consist of a monomethylated uronic acid combined with a series of d-xylan groups. The largest of these hemicelluloses contains approximately nineteen xylan groups in the molecule while the smallest contains as few as eight xylan groups. It appears that a mixture of hemicellulose molecules varying in size from approximately ten to nineteen xylan groups exists in the wood. During the process of isolation and purification some of these are hydrolyzed to still smaller molecules. The hemicelluloses which are colored blue or pink by iodine solution apparently contain anhydroglucose groups in the xylan chain. They seem to be intermediate products in the transformation of starch or dextrin to hemicelluloses. When all the facts are considered,

it appears that the same hemicelluloses are present in all the woods which do not contain starch and that these are formed from starch or dextrin.

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PURIFICATION OF THYROTROPIC HORMONE OF THE ANTERIOR PITUITARY*

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The secretion by the pituitary of a substance with specific stimulating effect on the thyroid gland has been demonstrated by the work of many investigators. While the biological properties of the thyrotropic hormone have been amply studied, it is surprising that but a few attempts have been made to purify and free the hormone from other active components of this gland. Preparations of varying potency have been described (1–26). In most cases, however, the exact procedures followed or the biological and chemical properties of the final product have not been stated adequately; in other cases new methods of assay have been employed but not correlated with preceding ones.

Method of Assay

Histological changes in the thyroid of young guinea pigs were early found to constitute a sensitive criterion for the detection of thyrotropic hormone (1, 27–29). Junkmann and Schoeller evaluated the potency of their fractions in a quantitative manner by means of a unit which they defined on the basis of this reaction (4, 5). This method of assay has since been used by many investigators. Others workers have based their evaluation of thyrotropic potency on the weight increase (20, 30) or iodine content

(31–33) of guinea pig thyroids; others on the effect of the hormone on the metabolic rate of hypophysectomized rats (14). More recently a new test has been suggested by Smelser (34, 35) based on the thyroid weight increase of day-old chicks. Smelser's work has been confirmed by other authors (36, 37). This method of bioassay, which has been well correlated with the histological guinea pig test, is now being widely used.

This test has also been employed in the present study and has yielded comparatively satisfactory results. Since the thyroids of individual chicks, however, vary considerably within one group, it is advisable to use as many birds per group as possible. With chicks from certain hatcheries groups of five, as suggested by Smelser, were found adequate, while with other chicks it was necessary to use larger groups, as was noted by Bergman and Turner (36). Using eight chicks per group, we have tested each fraction with several groups, and have used as many as forty chicks for more important preparations. Bergman and Turner have observed a sex difference in the response of chicks to thyrotropic fractions. We have also found this to be the case with chicks from certain hatcheries; whereas in those chicks which have been used for most of the present work there was no marked difference between the sexes. This has made it possible to use groups containing both male and female chicks.

Since quantitative work is greatly facilitated by the definition of a unit, the total dosage which in 6 days causes a 33 per cent increase in thyroid weights over that of the controls has been selected as a unit. This in chicks in groups composed of both sexes corresponds to the Bergman and Turner units of 50 per cent increase in male and 20 per cent increase in female chicks. Injections are performed subcutaneously, 0.2 cc. daily, for 5 days, with autopsies 24 hours after the last injection—conditions corresponding to those advocated by Smelser. The curves obtained when thyroid weight increase (up to 200 per cent) is plotted against the logarithm of the doses are generally straight lines. The slope of these lines was about the same for our preparations in the various stages of purification (1 unit according to definition causing 33 per cent increase, 2 units 92 per cent, 3 units 125 per cent, etc.). This slope can therefore be used in interpolating from a given response the amount of hormone corresponding to 1 unit.

In conjunction with the weight test in day-old chicks, we also

recently employed a modification of the test of Stimmel and McCullagh (31–33); *i.e.*, the decrease in iodine content of the thyroid gland of the injected animals.   Using guinea pigs, these authors defined as a unit a 50 per cent drop in the iodine content per thyroid lobe and stated that the decrease was parallel to the dose only up to about 2 times the unit; a minimum was then reached and further increase in dosage had no reliable effect on the residual iodine in the gland.   When we used McCullagh's method of analysis to determine the iodine content of groups of pooled chick thyroids following injection with thyrotropic hormone, similar effects were noted.   The test based on iodine decrease, however, is more sensitive than the weight test, so that less hormone is required to cause a 50 per cent drop in iodine content than to cause a 33 per cent gain in thyroid weight.   Thus the iodine content of thyroids, only slightly increased in weight (less than 33 per cent), may serve to differentiate between beginning stimulation and biological variation around the control value.   In agreement with Stimmel and McCullagh, no consistent parallelism could be detected between high doses and iodine content in the stimulated gland.

Correlation of Different Methods of Assay

In order to correlate different methods of assay with the day-old chick test (34, 35) the same preparation of thyrotropic hormone (Preparation O_V-20-II-III, 1 chick unit = 0.011 mg.) was tested in guinea pigs, pigeons, hypophysectomized rats, and 4 day-old chicks.

Histologically detectable thyroid stimulation of seven out of ten of the injected guinea pigs (150 gm.) was obtained with a total of 4 chick units of this preparation administered for 3 days, subcutaneously, according to the method of Junkmann and Schoeller (4, 5).   Rowlands and others (20, 30) suggest as a unit a 100 per cent increase of thyroid weights of 200 gm. guinea pigs, injected subcutaneously over a period of 5 days.   When assayed by this method, 9 chick units caused a 55 per cent increase; by means of the dose-response curve given by the above authors, it can be calculated that 17 chick units are equivalent to 1 of their guinea pig units.

When thyrotropic hormone was injected into 31 to 33 day-old

pigeons subcutaneously or intramuscularly for 4 days, about 2 to 4 chick units were required to cause histologically detectable stimulation. When this preparation was assayed in immature hypophysectomized female rats (1 week postoperative), a histologically detectable response of the thyroids following either subcutaneous or intraperitoneal injections for 3 days was obtained with a total of 9 chick units.

It was recently proposed to define as a unit the minimal dose required to cause histological stimulation of the thyroids of 8 day-old chicks, which had been injected twice daily for 4 days

TABLE I

*Comparison of Various Methods of Assaying Thyrotropic Hormone**

Test animal	Type of test	Approximate potency per mg. protein	Sensitivity†
		units	
Chicks	Weight	90	1.0
	Histological	90	1.0
Guinea pigs	Weight	5	0.06
	Histological	20	0.2
Hypophysectomized rats	"	10	0.1
Pigeons	"	30	0.3

* The standard preparation used for these various standardizations was Preparation Ov-20-II-III.

† The sensitivity of each test is relative to the chick thyroid weight test.

(38). We found that this unit was approximately the same as the one employed in the present investigation, based on the work of Smelser and others. In general, it can be said that the above findings summarized in Table I agree with the comparative sensitivities of the various methods of assay recorded in the literature.

Preparation and Purification

Although two methods have been recorded in the literature leading to a good recovery of purified thyrotropic preparations (4, 5, 23), both these methods were found to have certain disadvantages. The following procedure, which is relatively simple,

was regularly found to give good results and has led to a further purification of thyrotropic hormone.

Procedure[1]

Step 1—1 kilo of acetone-desiccated beef anterior pituitaries (4 to 5 chick units[2] per mg. of nitrogen) ground fine, was extracted twice for about 14 hours with stirring with 10 liters of 0.25 per cent acetic acid-1 per cent sodium chloride. Both extracts were centrifuged and the supernatants combined. Yield 23.2 gm. of nitrogen,[3] 23 units per mg. of nitrogen.

Step 2—The combined supernatants were precipitated with an equal quantity of acetone added with stirring. The solution was allowed to stand about 14 hours; the precipitate was collected and extracted twice, about 14 hours each time, with 4 liters of 1 per cent sodium chloride. Both extracts were centrifuged and the supernatants combined. Yield 4.8 gm. of nitrogen, 49 units per mg. of nitrogen.

Step 3—The combined supernatants were brought to 0.3 saturation with ammonium sulfate.[4] The precipitate was collected by centrifugation, dissolved (evenly suspended) in 1200 cc. of water, and again brought to 0.3 saturation with ammonium sulfate with formation of precipitate (Fraction A). The combined supernatants 0.3 saturated with ammonium sulfate were brought to 0.6 saturation with solid ammonium sulfate. The precipitate was collected, dissolved in 2 liters of water, and the solution brought to 0.3 saturation with ammonium sulfate with formation of a slight precipitate (Fraction AB). The supernatant from this second 0.3 saturated precipitate was brought to 0.5 saturation with ammonium sulfate. This precipitate (Fraction B) was collected, redissolved, and dialyzed against 1 per cent NaCl. Yield 0.86 gm. of nitrogen, 171 units per mg. of nitrogen.

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[1] All of the data given below represent an average of the results of ten repetitions of the preparation.

[2] Calculated from standardizations of thoroughly ground, evenly suspended fresh gland mash (of which 7.5 mg. corresponded to 1 unit).

[3] Determined by micro-Kjeldahl analysis by Mr. W. LaSalle.

[4] Unless otherwise stated, a saturated ammonium sulfate solution was used throughout to raise the salt concentration.

Step 4—To the dialyzed 0.5 saturated with ammonium sulfate fraction (Fraction B), diluted to 4.0 mg. of protein per cc, acetone was added to a concentration of approximately 39 per cent with stirring. The solution was allowed to stand 15 minutes and Precipitate I centrifuged off. The supernatant was poured into 8 to 10 times its volume of cold acetone, the solution standing ½ hour. Precipitate II was collected and dissolved immediately in 1 per cent NaCl. Yield 0.36 gm. of nitrogen, 480 units per mg. of nitrogen.

The entire procedure including acetone desiccation of the glands has been carried out at 0–5°; the organic solvents were always used at −10°.

Discussion of Steps in Procedure

Step 1—In agreement with observations of other authors (20, 39) anterior lobes of beef pituitaries were found to yield about twice as much thyrotropic potency as sheep glands and hence were used routinely.

While all aqueous extracts, ranging from pH 4 to 9, led to about the same recovery of thyrotropic activity, certain of these showed disadvantages. Extracts of fresh glands, whether acid, alkaline, or saline, are rather difficult to handle. Powdered, acetone-desiccated glands extracted with alkaline solutions are troublesome, inasmuch as they do not centrifuge well. Acid extractions of such acetone powder, as used by both Junkmann and Lambie, seemed the most effective procedure, yielding the same number of units in less extracted material. However, the second and third extracts did not yield clear solutions upon centrifugation. This disadvantage was avoided by extracting acetone-dried glands with acid saline solution.

Two extractions were found sufficient, the third extract containing only about 5 per cent and the residue 3 per cent of the total activity.

Step 2—The addition of acetone to the acid saline extract leads to a precipitation of most of the active principle, while the supernatant retains 59 per cent of the total nitrogen with less than 10 per cent of the thyrotropic potency. From the precipitate 65 per cent of the hormonal activity can easily be recovered by two extractions. A third extract as well as the entire residue contains only 6 per cent of the activity.

Step 3—Ammonium sulfate fractionation has led to further purification of the hormone. The first fraction 0.3 saturated with ammonium sulfate (Fraction A), comprising 15 per cent of the starting material, contains less than 6 units per mg. of nitrogen. The second precipitate 0.3 saturated with ammonium sulfate (Fraction AB) varies in amount, being proportionately smaller when smaller quantities (*e.g.*, 250 gm.) of acetone powder have been used for extraction. On the average, this fraction contains 5 per cent of the nitrogen and 29 units per mg. Fraction B precipitating between 0.3 and 0.5 salt saturation is the most potent, containing 1 unit in 0.030 to 0.050 mg. of protein.[5] When the supernatant from Fraction B is brought to 0.6 saturation with ammonium sulfate, another precipitate (Fraction C) is obtained which is comparatively active (100 units per mg. of nitrogen) but represents only 5 per cent of the total protein. The residual

TABLE II
Ammonium Sulfate Fractions

Fraction	A	AB	B	C	D
Units per mg. N...	6	29	171	100	10
Yield in unitage, %.....	1.8	3.0	62.5	10.2	9.6

protein (Fraction D) in the supernatants 0.6 saturated with ammonium sulfate (47 per cent) contains only about 10 units per mg. of nitrogen. The above data on the ammonium sulfate fractionation are summarized in Table II.

Recent experiments have indicated that the same ammonium sulfate procedure can be used to fractionate acid saline extracts of the desiccated glands directly, without the acetone precipitation (Step 2). B fractions thus obtained have about half the potency of those prepared by the method outlined above but the recovery of hormonal activity in fractions obtained by this simplified method appeared to be more complete.

Step 4—Following the ammonium sulfate fractionation, separation by means of organic solvents, preferably acetone, leads to

[5] The nitrogen percentage (13.9 per cent) of this protein fraction was determined by evaporation of solutions which had been dialyzed against distilled water.

further purification of the hormone. It is difficult to give the exact conditions for the removal of a maximum amount of inert protein in the first precipitate (Precipitate I) so that it is advisable in each case to determine in preliminary experiments the acetone concentration which will cause a precipitation of about 50 per cent of the protein. It was observed, furthermore, that the length of time during which preparations of more than 150 units per mg. of nitrogen were left in contact with organic solvents influenced strongly the recovery of potency. Great losses were occasionally incurred when the specified time limits were not followed in fractionating these more potent preparations. It was also found that repetition of procedures involving the use of organic solvents led to loss of potency. When these precautions are taken into account, Precipitate I will contain approximately 50 per cent of the nitrogen, but with little potency (less than 1 unit per 0.01 mg. of nitrogen), while Precipitate II will be twice as potent as the ammonium sulfate fraction (Fraction B) and will contain 1 unit in 0.0016 to 0.0035 mg. of nitrogen. This product is approximately 3 times as active as that obtained by the best preparation methods recorded in the literature.

More purified fractions have been obtained with alcohol as the fractionating agent following the above acetone procedure. 50 per cent alcohol concentration was found to cause the precipitation of inert protein, while from the supernatant highly potent fractions containing about 1000 units per mg. of nitrogen could be obtained by precipitation with excess acetone. As indicated above, however, the total recovery of potency in these fractions was often low, even if the same precautions were observed in this fractionation as in the previous step.

Properties

The anterior pituitary protein associated with thyrotropic activity is relatively soluble in distilled water and easily soluble in dilute salt, acid, or alkaline solutions. This extreme solubility may account for the fact that experiments to precipitate this protein isoelectrically even from very concentrated solutions over the range of pH 4 to 9 have been unsuccessful.

Chemical analyses have shown this protein to contain 13 per

cent nitrogen, 3.5 per cent carbohydrate,[6] and 2.5 per cent glucosamine.

Cysteine treatment, under conditions favorable for the reduction of —S—S— bonds, was found to inactivate the hormone.

Ketene[6] treatment for 5 minutes at 22° in acetate buffer of pH 5.6 caused considerable loss of activity.

Contamination with Other Pituitary Hormones

Lactogenic Hormone—Preliminary tests of comparatively crude thyrotropic fractions by intrapectoral or subcutaneous injection into pigeons have shown these preparations to be very poor in crop gland-stimulating activity. Purified fractions were therefore tested by the local crop test (40) by which as little as 0.0001 mg. of lactogenic hormone can be detected. Thyrotropic Preparation O_V-20-II-III (1 unit = 0.011 mg. of protein) caused a minimal response when thus tested at 0.400 mg., but not at 0.100 mg. This shows that 40 units of thyrotropic hormone contain 1 "local" unit of lactogenic hormone. This actually means that the thyrotropic preparation contains only 0.025 per cent lactogenic hormone.

Adrenocorticotropic Hormone—Since the usual method of assay for this hormone necessitates much material, the recently developed, more sensitive test in 4 day-old rats (41) was here employed. When 7.2 mg. of Preparation O_{VI}-64-CII (1 unit = 0.012 mg. of protein) were injected, only 22 per cent increase in adrenal weights resulted, whereas 0.25 mg. of the best corticotropic preparation causes a 50 per cent increase. This would indicate a contamination of less than 3 per cent with this hormone.

When a total dose of 160 to 300 units of different thyrotropic preparations was injected over a period of 10 days into hypophysectomized rats, the weights of the adrenals of these animals were not significantly increased, and there was only doubtful histological repair of the cortex. However, 800 units (1 mg. daily, Preparation O_{VI}-64-CII) caused 36 per cent increase in the weight of adrenals, the cortex of which was at the same time re-

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[6] The carbohydrate determination and ketene treatment were kindly carried out by Dr. C. H. Li of this department.

paired. A comparable effect on the adrenals was given by 0.2 mg. daily of growth hormone preparations (globulin fraction of alkaline extracts of beef pituitaries).

Growth Hormone—As in previous assays, the most sensitive test was also employed for this hormone. When injected into ten hypophysectomized rats for 10 days, 0.20 mg. daily of Preparation O_{VI}-64-CII caused no gain in body weight, whereas 5-fold this dose (in five rats) caused 13 gm. growth. This growth corresponds to about 1 unit of growth hormone. Since 0.010 mg. of the most purified growth preparations available cause a similar effect, this would correspond to a contamination of about 1 per cent with such growth preparations.

Gonadotropic Hormones—The content of follicle-stimulating hormone in purified thyrotropic preparations was tested in immature hypophysectomized female rats. 100 units of such fractions were found to contain 1 unit of follicle-stimulating hormone. Since 0.004 mg. of the best follicle-stimulating hormone fractions contain 1 unit, these thyrotropic preparations are contaminated with only 0.4 per cent of this follicle-stimulating protein.

The content in purified thyrotropic preparations of the interstitial cell-stimulating or luteinizing[7] factor was of particular interest in view of recent claims that thyrotropic activity is a property of this component of the gonadotropic complex, rather than due to a separate entity (38, 44, 45). Three purified thyrotropic fractions were therefore tested for the interstitial cell-stimulating factor in the routine manner in hypophysectomized female rats. Two of these showed beginning repair of the ovarian interstitial tissue at levels of 0.100 to 0.150 mg. Since purified interstitial cell-stimulating hormone preparations cause repair a 0.010 mg., the above finding indicates these thyrotropic preparations to be about one-tenth as potent in this respect as purified interstitial cell-stimulating hormone.

Purified thyrotropic preparations were also tested for antagonistic and luteinizing properties. Antagonism was measured by th usual method of combining the solutions to be tested with standard amount of pregnant mare serum gonadotropin and in

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[7] The relationship between the interstitial cell-stimulating properties described from this laboratory (42) and the luteinizing properties defined by Fevold (43) will be discussed in a future publication.

jecting this mixture intraperitoneally. The tested thyrotropic preparations were found to be about one-fifth as active in this regard as the "antagonist" preparations.

Luteinizing potency was estimated according to Fevold (43) by the ability of solutions to augment the ovarian weights secured by a standard amount of follicle-stimulating hormone in the female and to stimulate increase of seminal vesicle weight in the male rat. In these respects, the thyrotropic preparation proved about one-tenth as active as one of our interstitial cell-stimulating or luteinizing preparations (Preparation III-F33CS).

In view of the relatively low content of interstitial cell-stimulating (luteinizing) factor of highly active thyrotropic preparations, it seemed unlikely that these diverse effects were due to one hormone. Further investigation of this question was facilitated by the fact that highly purified interstitial cell-stimulating hormone preparations were kindly supplied by Dr. C. H. Li of this department. These (Preparations L79C, L110B) contained 100 units of both interstitial cell-stimulating and "antagonist" potency per mg. They were obtained by different methods and were found to be homogeneous in the electrophoresis apparatus. Both these preparations were found to contain less than 1 chick unit of thyrotropic hormone at the highest level tested (0.5 mg.), which corresponds to 50 interstitial cell-stimulating units. It has been suggested (45) that the antagonistic effects given by interstitial cell-stimulating fractions may be due to the thyroid stimulation caused by these fractions. It is now evident that this mechanism cannot explain the high antagonistic potency of purified interstitial cell-stimulating fractions, since these have been shown to cause no such thyroid stimulation at 50-fold the level which shows 1 unit of antagonism.[8]

To compare the properties of a thyrotropic with those of a crude interstitial cell-stimulating (luteinizing) preparation in the same test object, the effect of these fractions on the weights of thyroids and testes in day-old chicks has been investigated.

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[8] The demonstration of antagonism of a pituitary fraction against pregnant mare serum gonadotropin in thyroidectomized rats by Evans and associates (46) had already established the fact that this type of antagonism could not be mediated by the thyroid.

The injection of 0.2 mg. of thyrotropic Preparation O_V-20-II-III led to 250 per cent increase in thyroid and only 72 per cent increase in testis weight.　On the other hand, an equal amount of a gonadotropic preparation (No. III-F33CS) gave 130 per cent increase in testis with only 35 per cent increase in thyroid weight.

From all these experiments it becomes evident that, while not being identical with any component of the gonadotropic complex, the best preparations of thyrotropic hormone are contaminated with as much as 10 per cent of interstitial cell-stimulating factor.[9]

SUMMARY

1. Five methods of assay of thyrotropic hormone have been correlated with the test based on the gain in weight of chick thyroids.　Details of the chick test have been discussed and a unit defined.

2. A method of preparation and purification of the thyrotropic factor of the anterior pituitary has been described in detail. The final product represents a hundredfold purification with recovery of about one-third of the total thyrotropic activity of the starting material.

3. Some of the properties of the active protein have been reported.　The degree of contamination of this protein with other pituitary hormones has been investigated.　It was found to contain about 10 per cent of the interstitial cell-stimulating hormone but very little lactogenic, adrenocorticotropic, growth-stimulating, or follicle-stimulating hormones.

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[9] The conclusion that thyrotropic hormone and interstitial cell-stimulating (luteinizing) hormone cannot be identical has also been reached by other investigators on the basis of selective enzymatic inactivation (47).

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AN ASSAY METHOD FOR PANTOTHENIC ACID

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Because of its general biological importance a method for the
satisfactory quantitative determination of pantothenic acid is
desirable. At present there is no immediate promise that pan-
tothenic acid can be determined by colorimetric or other physical
or chemical means and it appears probable that biological testing
will have to be relied upon.

The available methods involve measuring the growth responses
of a yeast (1, 2), a lactic acid organism (3, 4), or of chicks (5, 6).
The last possibility is, of course, cumbersome and inconvenient
as compared with the first two and might find use only in cases
in which the pantothenic acid could not be extracted quantita-
tively from tissues to be examined.

While either the yeast method or the bacterial method gives
satisfactory results when concentrates are tested, their application
to crude tissue extracts has never been adequately studied.

In studies preliminary to the present one it was found that the
yeast method gives results which are subject to error even when
materials are tested at low dosage levels as recommended (7).
The bases for errors lie (a) in the adverse effect of toxic substances
(1), (b) in the stimulating effect of nutrilites unrelated to pan-
tothenic acid (1), (c) in the fact that β-alanine, a cleavage product
of pantothenic acid (also of carnosine and anserine), is sufficiently
stimulative to interfere appreciably (7).

We have found it possible to modify the bacterial test so that
it yields results from tissue extracts which we believe to be reliable.
The organism chosen does not in general appear to be affected
adversely by toxic substances which are likely to be present in

tissue extracts (8). The alkali-treated basal medium previously used (3, 4) has been modified so that other stimulative (but non-essential) nutrilites apparently present in crude extracts are present (pantothenic acid is destroyed by alkali treatment). β-Alanine which is present as a cleavage product is without effect (4).

There are alternative methods which can be used to destroy pantothenic acid specifically and in case of special materials which offer difficulties (urine is an example) we have been able to obtain satisfactory and consistent results by placing in the basal medium a sample of the same urine, treated to eliminate the effect of the pantothenic acid originally present.

EXPERIMENTAL

Organism—The organism used is *Lactobacillus casei* ε, and is the same strain as that used in the quantitative determination of riboflavin as described by Snell and Strong (8).[1] Stock cultures of the organism are carried as stabs in yeast extract-glucose-agar (1 per cent glucose, 1 per cent yeast extract, 1.5 per cent agar). These stabs are prepared from previous stock cultures at monthly intervals. After transfer, cultures are incubated at 37° for 24 to 48 hours, then held in the refrigerator. Inoculum for assay tubes is prepared by transfer from the stock culture to a sterile tube of the basal medium (described below) to which an excess of pantothenic acid (or pantothenic acid-containing extract; *e.g.*, yeast extract) has been added. The inoculum is incubated at 37° for 24 hours before use. For tests on successive days, inoculum for the following day may be prepared from that of the preceding day. To minimize chances of contamination and bacterial variation, a return to stock cultures should be made about once a week.

Basal Medium—The pantothenic acid-free, basal medium used is a modification of that used by Snell, Strong, and Peterson (3, 4) for the determination of pantothenic acid, and by Snell and Strong (8) for the determination of riboflavin. It contains alkali-treated peptone 0.5 per cent, glucose 1 per cent, sodium acetate

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[1] Cultures of this organism may be obtained from the American Type Culture Collection, Georgetown University Medical School, Washington, where it is listed as No. 7469.

0.6 per cent, alkali-treated yeast extract equivalent to 0.1 per cent yeast extract, acid-hydrolyzed casein equivalent to 0.2 per cent casein, cystine 0.01 per cent, riboflavin 0.01 mg. per cent, and inorganic salts. The addition of hydrolyzed casein and alkali-treated yeast extract to the medium previously used (4) increases the specificity of the response to pantothenic acid. The constituents of the medium are prepared as follows:

Alkali-Treated Peptone—40 gm. of Bacto-Peptone (Difco) in 250 cc. of water are treated with 20 gm. of sodium hydroxide dissolved in 250 cc. of water. The mixture (1 N in NaOH) is allowed to stand at room temperature for 24 hours. The sodium hydroxide is neutralized with glacial acetic acid (27.9 cc.), 7 gm. of anhydrous sodium acetate (11.6 gm. of the trihydrate) are added, and the mixture is diluted to 800 cc. The solution may be preserved under toluene.

Cystine—A solution of cystine hydrochloride containing 1 mg. of cystine per cc. is prepared and kept under toluene.

Alkali-Treated Yeast Extract—A solution of 20 gm. of Difco yeast extract in 200 cc. of 0.5 N sodium hydroxide is autoclaved at 15 pounds pressure for 30 minutes. The solution is neutralized with glacial acetic acid, autoclaved an additional 10 minutes to coagulate precipitated proteins, and filtered. The volume is adjusted to 200 cc. (100 mg. per cc.) and the solution preserved under toluene.

Acid-Hydrolyzed Casein—50 gm. of technical casein are mixed with 250 cc. of 25 per cent sulfuric acid. The mixture is autoclaved for 10 hours at 15 pounds pressure. The sulfuric acid is removed with barium hydroxide (341 gm. of $Ba(OH)_2 \cdot 8H_2O$). Any excess barium ion is carefully removed with sulfuric acid, and the pH adjusted to 7.0 with sodium hydroxide. The solution is made to a volume of 450 cc., autoclaved for 10 minutes, and filtered. It is preserved under toluene.

Inorganic Salts—Solution A consisted of 25 gm. of potassium monohydrogen phosphate and 25 gm. of potassium dihydrogen phosphate dissolved in 250 cc. of water.

Solution B consisted of 10 gm. of magnesium sulfate heptahydrate, 0.5 gm. of sodium chloride, 0.5 gm. of ferrous sulfate heptahydrate, and 0.5 gm. of manganese sulfate tetrahydrate dissolved in 250 cc. of water. Salts precipitate from Solution B

when it stands in air; it need be renewed only when a uniform suspension can no longer be secured by shaking.

5 cc. of Solution A plus 5 cc. of Solution B contain inorganic salts for 1000 cc. of basal medium.

Procedure—Assays are carried out in 6 inch chemical or bacteriological test-tubes. These are conveniently supported by a wire or metal rack, which may be autoclaved. If, for example, ten assay tubes were to be inoculated from stock solutions prepared as described above, the following amounts would be used: 10.0 cc. of alkali-treated peptone, 2.0 cc. of casein hydrolysate, 1.0 cc. of alkali-treated yeast extract, 10.0 cc. of cystine hydrochloride solution, 0.5 cc. of inorganic salts (Solution A), 0.5 cc. of inorganic salts (Solution B), 1.0 gm. of glucose, 1.0 cc. of riboflavin solution (10 γ per cc.).[2]

The mixture is adjusted to a pH of 6.6 to 6.8 and diluted to 50 cc. This is double the concentration of the final medium. The standard pantothenic acid preparation and samples for analysis are added to the tubes. Volumes up to 5 cc. may be used. The contents of all tubes are then diluted if necessary to 5 cc. with water; then 5 cc. of the basal medium prepared as above are pipetted into each tube. Tubes are plugged with cotton and sterilized in the autoclave at 15 pounds steam pressure for 15 minutes. After cooling to room temperature, tubes are ready for inoculation.

The cells from a 24 hour culture of inoculum grown as described previously are centrifuged out (aseptically) and resuspended in approximately double the original volume of 0.9 per cent saline solution. 1 drop of the resulting heavy suspension of organisms is added to each assay tube. Variation in the size of individual drops used for inoculation causes inappreciable errors in the final results. Aseptic precautions should be observed throughout. Tubes are now incubated at 37° for the growth period (see below).

Measurement of Response to Pantothenic Acid—Two methods for measuring the response to added pantothenic acid are applicable. (*a*) The lactic acid produced during growth may be titrated after 72 hours incubation. Because of the color of the medium

[2] A solution of riboflavin in 0.02 N acetic acid is prepared and kept under toluene in the refrigerator. Unnecessary exposure to light should be avoided, and the fresh solution prepared at frequent intervals.

and its buffer capacity, a glass electrode is most suitable for this titration; or the medium may be diluted and brom-thymol blue used as an indicator for direct titration. (*b*) The turbidity produced by the organisms after 24 hours incubation may be quantitatively determined in a thermoelectric turbidimeter such as that described by Williams, McAlister, and Roehm (2) or by use of a suitable photoelectric colorimeter, such as that described by Evelyn (9). Because of the saving in time, the turbidimetric
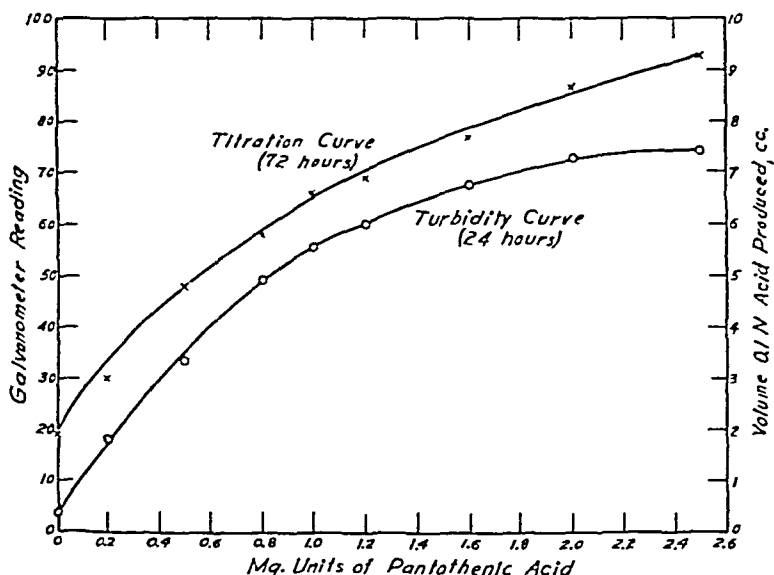
FIG. 1. Response of *Lactobacillus casei* ε to added pantothenic acid concentrate.

method (with the thermoelectric turbidimeter) has been used in preference to the titrimetric method in this laboratory, and unless stated otherwise, all data cited were obtained by this method. Care must be taken to suspend sedimented organisms by shaking before turbidity measurements are made.

Results

Response of Lactobacillus casei ε to Pantothenic Acid—The response to added pantothenic acid concentrate as determined by

both methods is shown in Fig. 1.   Nearly direct proportionality exists between amounts of added pantothenic acid and ordinate readings from 0 to 1.2 mg. units [3](7) of pantothenic acid.   Values below 0.05 mg. unit are not reproducible, and the range 0.05 to 1.2 mg. units is used for assay purposes.   A standard curve similar to Fig. 1 must be obtained with each set of assays; the samples for assay are set up at increasing levels of concentration estimated to contain between 0.1 and 1.2 mg. units of pantothenic acid; the pantothenic acid content of each "unknown" tube is then read from the standard curve.   The agreement of assay figures when calculated from the different levels is one of the strongest indications that the test is specific.

TABLE I

Recovery Experiments with Pantothenic Acid Concentrates

Pantothenic acid concentrate	Turbidimetric method		Titrimetric method	
	Found	Recovery	Found	Recovery
mg. units per cc.*	mg. units per cc.	per cent	mg. units per cc.	per cent
0.22	0.23	105	0.24	109
0.31	0.29	94	0.33	106
1.33	1.28	96	1.39	105
2.38	2.40	101	2.52	106

* As originally determined by the yeast test.

Application of Method—The assay of purified concentrates of pantothenic acid offers no difficulty.   In Table I results of such assays made by both turbidimetric and titrimetric methods are compared.

Extracts for assay of natural materials are prepared by autoclaving the finely ground (homogenization as described by Potter and Elvehjem (10) is effective) material at neutrality with a large volume of water for 30 minutes at 15 pounds steam pressure. Wherever suitable, autolysis under benzene is allowed to take place preliminary to autoclaving.   This frees additional pantothenic acid from many tissues.   Where necessary fullers' earth or kieselguhr may be used to clarify extracts without decreasing their pantothenic acid content.

Sample results are shown in Table II.   It will be noted that

[3] 1 mg. unit = 0.08 γ of calcium (+)-pantothenate (Merck).

TABLE II

Pantothenic Acid Content of Natural Materials

The roman numerals represent the assay numbers.

Material assayed	Amount assayed			Pantothenic acid added per mg. sample			Pantothenic acid found per mg. sample			Recovery of added pantothenic acid		
	I	II	III	I	II	III	I	II	III	I	II	III
	mg.	mg.	mg.	mg. units	mg. units	mg. units	mg. units	mg. units	mg. units	per cent	per cent	per cent
Yeast extract	0.3	0.2	0.1	0	0	0	3.6	3.6	3.7			
	0.2	0.1	0.05	1.0	5.0	11.7	4.6	0.0	15.2	100	108	98
Alkali-treated yeast extract	0.3	0.2	0.1	0	0	0			*			
	0.3	0.2	0.1	1.07	2.50	5.00	1.07	2.40	4.80	100	96	96
Liver extract	0.2	0.10	0.10	0	0	0	3.4	3.4	3.3			
	0.20	0.10	0.10	1.00	1.25	5.00	4.5	4.75	8.4	110	108	100
Alkali-treated liver extract	0.20	0.16	0.10	0	0	0			*			
	0.20	0.16	0.10	2.50	3.13	5.00	2.45	2.94	4.80	98	94	96
Whey	3.0	5.0	8.0	0	0	0	0.080	0.082	0.086			
	10.0			0	0	0.05	0.089					
	3.0	5.0	8.0	0.20	0.10		0.283	0.188	0.131	100	104	94
	10.0			0.03			0.115			103		
Alkali-treated whey	5.0	10.0	15.0	0	0	0			*			
	5.0	8.0	10.0	0.100	0.003	0.050	0.110	0.065	0.051	110	103	102

* Values obtained on interpolation on the curve were below 0.05 mg. unit, the lower limit used for assay purposes.

the following requirements indicative of specificity were met. (*a*) Assay values calculated from different dosage levels of the sample agree. (*b*) The activity of the sample as indicated by the test is destroyed by alkaline hydrolysis, which inactivates pantothenic acid by cleavage. (*c*) Additions of pantothenic acid concentrate to the untreated natural materials and to the alkali-treated materials are quantitatively recovered.

Considerable data resulting from the application of the assay method (in approximately its present form) to chick tissues were recently presented from this laboratory (11) and compared with similar assays made by the yeast method.

When assayed as described above, certain materials give assay figures which do not agree satisfactorily when calculated from different levels; recoveries of added pantothenic acid concentrate were also seriously in error. Most important of these materials was urine; others were oysters and mushrooms. In each case the difficulties could be eliminated by adding to the basal medium a specific supplement, prepared by acid treatment of the sample in question (like alkali, acid inactivates pantothenic acid by cleavage). In the case of urine this supplement was prepared by making the urine 0.5 N with sulfuric acid, and autoclaving at 15 pounds steam pressure for 20 minutes. Sulfuric acid was exactly removed with barium hydroxide. With oysters and mushrooms the supplements were prepared by similar treatment of aqueous extracts of these materials equivalent to approximately 100 mg. of fresh tissue per cc. The supplement is added to the basal medium in approximately 10 times the highest concentration used for the assay sample (*e.g.*, acid-treated urine equivalent to 0.2 cc. of original urine per tube in the case of urine assays, Table III).

The application of this modification to assays of urine and to extracts of oysters and mushrooms is shown in Table III. Assay values at different dosage levels are in excellent agreement, and satisfactory recovery of added pantothenic acid was obtained.

Such a scheme is applicable wherever assay values found with the simpler medium do not agree satisfactorily at different levels. Such an acid-treated supplement may be valuable for regular addition to the medium in routine assays; *e.g.*, of urine.

That none of the known growth substances (or related materials)

was responsible for the results observed with these extracts was shown by separate experiments which it seems unnecessary to report in detail. Additions of uracil, nicotinamide, β-alanine, ethanolamine, choline, creatine, riboflavin, biotin, thiamine, vitamin B_6, inositol, pimelic acid, indole-butyric acid, or trauma-

TABLE III

Pantothenic Acid Content of Urine, Oysters, and Mushrooms Assayed in Presence of Acid-Treated Extracts

The roman numerals represent the assay numbers.

Material assayed	Amount assayed			Pantothenic acid added per mg. sample			Pantothenic acid found per mg. sample			Recovery of added pantothenic acid		
	I	II	III	I	II	III	I	II	III	I	II	III
	mg.	mg.	mg.	mg. unit	mg. unit	mg. unit	mg. unit	mg. unit	mg. unit	per cent	per cent	per cent
Urine	10.0	16.0	20.0	0	0	0	0.028	0.027	0.028			
	4.0	10.0	16.0	0.200	0.050	0.013	0.23	0.079	0.041	100	102	100
Oysters	5.0	10.0	15.0	0	0	0	0.052	0.047	0.047			
	20.0			0			0.047					
	5.0	10.0	15.0	0.140	0.050	0.020	0.196	0.102	0.068	106	108	100
Mushrooms	1.5	3.0	4.5	0	0	0	0.18	0.18	0.17			
	6.0			0			0.18					
	•1.5	2.4	3.0	0.47	0.21	0.10	0.67	0.39	0.29	104	100	110

tin alone or in combinations did not interfere with the quantitative determination of added pantothenic acid.

DISCUSSION

The method described above is applicable only to extracts of tissues, and leaves untouched the question of the quantitative extraction of pantothenic acid from tissues. In our experience, the method recommended gives reproducible results, and more nearly quantitative extraction than any other tried. Quantitative extraction from tissues is made difficult by the lability of pantothenic acid to acid and alkali (3, 7), and its destruction by acidic acetone or alcohol (12).

SUMMARY

A biological assay method for determining the pantothenic acid content of tissue extracts is described which is rapid, accurate,

and specific.   This method, a modification of that used by Snell *et al.* (4, 8) for the determination of pantothenic acid and riboflavin, involves the response of *Lactobacillus casei* ϵ to pantothenic acid, and has been applied successfully to extracts of yeast, liver, milk, oysters, and mushrooms, to urine, and to chick tissues. Evidence as to its specificity in addition to that previously published is based upon the fact that different dosage levels yield results which are in agreement and the agent assayed is destroyed under conditions which destroy pantothenic acid.   Pantothenic acid (concentrates) added to tissue extracts is "recovered" quantitatively in the test.

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THE COMPARATIVE ACTION OF CHOLINE AND BETAINE IN EFFECTING THE REPLACEMENT OF METHIONINE BY HOMOCYSTINE IN THE DIET

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In a recent communication by du Vigneaud, Chandler, Moyer, and Keppel (1) it was reported that either choline or betaine would enable an animal to utilize homocystine or homocysteine in the diet in place of methionine. With these compounds, the white rat was able to grow on a diet which was free of methionine but which contained homocystine; whereas, in their absence, the animals were unable to grow. The obvious inference was drawn by these authors that, in the presence of choline or betaine, the body was able to methylate the sulfur of homocysteine to give methionine. An actual transfer of methyl groups was postulated. It was suggested that the reaction might be reversible and that methionine might be the precursor of choline in so far as furnishing the methyl groups is concerned. The possibility of methionine being involved in other methylations of the body was likewise pointed out (1). It is of interest in this connection that Borsook has recently found that in the presence of methionine an increased amount of guanidoacetic acid could be methylated by liver slices to form creatine (2).

The present communication deals with the relative potencies of choline and betaine in bringing about the utilization of homocystine. Preliminary work had given some indication that choline was more effective (1). This led to the tentative conclusion that betaine was probably effective in so far as it was converted to choline within the body. A more careful comparison of the effects of the two compounds was desirable. In order to rule out as far as possible the chance of bacterial conversion, it was decided

to compare the compounds, not only by administration *per os*, but also by parenteral administration.

<center>EXPERIMENTAL</center>

Young white rats were placed on a diet free of cystine and methionine in which the amino nitrogen was supplied by pure amino acids in a mixture modeled after that used by Rose and Rice (3). The diet had the following composition: amino acid mixture 23.6 (1), *dl*-homocystine 1.25, dextrin 24.15, sucrose 15.0, salt mixture (Osborne and Mendel (4)) 4.0, agar 2.0, and corn oil (Mazola) 30.0 parts. Irradiated ergosterol,[1] *dl*-α-tocopherol, and halibut liver oil (S. B. Penick and Company) were added to the corn oil in such amounts as to provide 20 U.S.P. units of vitamin D activity, 0.05 mg. of *dl*-α-tocopherol, and 200 U.S.P. units of vitamin A per rat per day. The halibut liver oil contained 90,000 U.S.P. units of vitamin A per gm., and the irradiated ergosterol in cottonseed oil 600,000 U.S.P. units per gm. The water-soluble vitamins were supplied by two pills daily, each having the following composition: thiamine chloride 10 micrograms, riboflavin 10 micrograms, nicotinic acid 10 micrograms, modified ryzamin-B (freed of choline by precipitation as the reineckate (1)) 12.5 mg., and dextrin 150 mg. With Litters I and III the modified ryzamin-B was omitted and 5 micrograms of vitamin B_6 were added to each pill. We had previously found that rats grew normally during the short periods used in these experiments when the ryzamin-B was omitted from the diet.

Rats placed on this basal diet containing homocystine failed to grow. Excellent growth resulted, however, when the proper mounts of either choline or betaine were administered. In order to compare the relative effectiveness of these two compounds in producing growth they were given at suboptimal or minimal levels. The method of administration was either in the pill with the water-soluble vitamins, or by subcutaneous injection twice daily.

Litter I was placed on the diet described above but without the modified ryzamin-B at approximately 3 weeks of age. After a 4 day preliminary period of adjustment to the diet and the experimental cages, two rats were given orally 10 and 25 mg. of choline chloride per day, respectively, two others were given equivalent amounts of betaine chloride, and one was allowed to continue without either supplement. The two rats receiving choline began to grow immediately and continued to gain weight
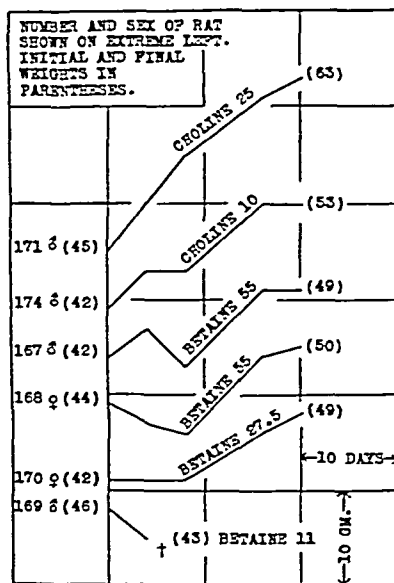
Fig. 1. Growth curves of Litter II showing the effect of various levels of betaine and choline, which are given in mg., administered *per os*.

(approximately 1 gm. per day) during the 16 days on these supplements. The control animal, and the two rats receiving betaine, all died within 7 days.

Another litter of rats, Litter II, was started at approximately 3 weeks of age and betaine was given at high levels. These animals were given modified ryzamin-B. Two rats were given orally 10 and 25 mg. of choline chloride per day, respectively, two were given equivalent amounts of betaine chloride, and two others were given 55 mg. of betaine chloride. The growth curves of

this litter are shown in Fig. 1 and the food consumption is given in Table I. All of the rats survived and gained weight except the one getting the smallest amount of betaine. The other rats receiving betaine, even at the 55 mg. level, showed poor growth compared with the choline-fed animals, particularly during the first 8 days of the experiment. Following this initial period, however, these rats showed a decided increase in the rate of growth and maintained levels only slightly inferior to those of the choline-

TABLE I

Litter No.	Rat No. and sex	Days	Daily supplement				Average daily food consumption
							gm.
II	167 ♂	1–20	55	mg. betaine chloride			3.2
	168 ♀	1–20	55	"	"	"	3.5
	169 ♂	1– 4*	11	"	"	"	
	170 ♀	1–20	27.5	"	"	"	2.5
	171 ♂	1–20	25	" choline	"		3.8
	174 ♂	1–20	10	"	"	"	3.6
III	115 ♂	1–40	25	"	"	"	6.0
	116 ♀	1–23	5	"	"	"	3.7
		23–40	50	"	"	"	3.9
	117 ♀	1–40	10	"	"	"	4.1
	118 ♀	1–40	No supplement				2.5
	120 ♂	1–40	27.5 mg. betaine chloride				4.6
	121 ♀	1–23	5.5	"	"	"	5.0
		23–32	55	"	"	"	4.9
	122 ♀	1–40	11	"	"	"	5.0

* Died.

fed animals. It may be noted, in contrast, that choline produced an immediate and sustained growth response.

The rats of Litter III were placed on the diet free of cystine and methionine with no supplement of modified ryzamin-B at approximately 5 weeks of age. Choline was given at 5, 10, and 25 mg. levels, and betaine to three other rats in equivalent amounts. These supplements were administered by subcutaneous injection twice daily. The growth curves for this litter are given in Fig. 2 and the food consumption is shown in Table I. The results show that choline was ineffective at the level of 5 mg. daily,

but produced moderate growth at the 10 mg. level, and very good growth at the 25 mg. level. Betaine was less effective at corresponding levels. It is to be noted that the principal difference between the effect of 25 mg. of choline chloride given to Rat 115
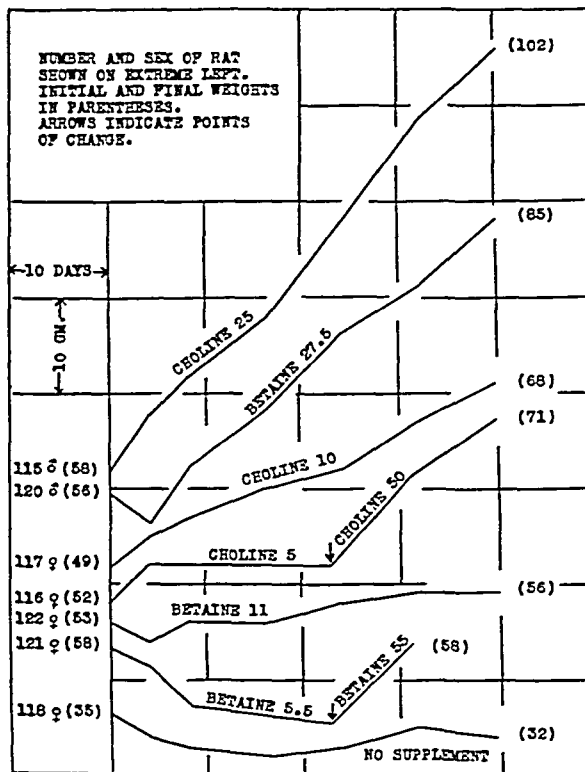
FIG. 2. Growth curves of Litter III showing the effect of daily subcutaneous injections of various levels (given in mg.) of choline and betaine.

and the effect of the equivalent amount of betaine given to Rat 120 was the delay of several days before growth began in the latter case.

DISCUSSION

The observations recorded in this paper demonstrate that betaine is less effective than choline in supporting growth of rats on a diet containing homocystine but free of cystine and

methionine. The difference is manifested principally in a delay of several days in the response of growth following the administration of betaine to deficient animals. When the rats are only 3 to 4 weeks old, this delay in action often results fatally. This is of interest in connection with the observations of Griffith and Wade (5) on the high mortality of young rats on a low choline diet. The mechanism for the effect of betaine in bringing about the transformation of homocystine to methionine appears to be absent or at least inefficient at first, but is fully developed during a period of several days after the compound is provided to the animals. This is true even when the compounds are injected.

One explanation of these results might be that choline is the compound which is actually used by the body for the methylation of homocystine but that betaine can be used after the organism acquires the ability to convert sufficient quantities of it to choline. Support for such a theory is also found in the fact that even after the first delay in action betaine is somewhat less effective than equivalent amounts of choline. This inequality of activity of the two substances could be accounted for by a less than quantitative transformation of betaine to choline.

It is realized, of course, that relative effects are only suggestive of such a conversion, and not proof, since both compounds could act directly with different inherent activities. The effect of different rates of destruction of the two compounds in the body must also be recognized as a factor in determining the amounts required to produce a given effect.

The bacterial flora are apparently not necessary agents in the use of choline and betaine for the conversion of homocystine to methionine, inasmuch as subcutaneous administration of the compounds is as effective as their inclusion in the diet. Since parenteral administration also does not alter the relative effectiveness of the two compounds, it may be justified to assume that the bacterial flora are not required for any interconversion that may occur.

SUMMARY

Choline has been found to be more effective than equivalent amounts of betaine in enabling an animal to utilize homocystine

in place of methionine. Both compounds are active in this respect whether administered *per os* or parenterally.

The authors wish to thank Miss Doris Flavelle for her worthy assistance in this investigation.

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RENAL EXCRETION OF HEXITOLS (SORBITOL, MAN-NITOL, AND DULCITOL) AND THEIR DERIVATIVES (SORBITAN, ISOMANNIDE, AND SORBIDE) AND OF ENDOGENOUS CREATININE-LIKE CHROMOGEN IN DOG AND MAN*

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Inulin is the only substance hitherto reported which fulfils the physiological specifications for the measurement of the rate of glomerular filtration in man (34). The only other substance advocated for this purpose is creatinine (28). In the dog, the exogenous creatinine clearance is identical with the inulin clearance, and both clearances are independent of plasma concentration, which facts are good evidence that, in this animal, both clearances are at the level of filtration. But in man, the exogenous creatinine clearance exceeds the simultaneous inulin clearance by 40 per cent or more (19, 24, 30, 33), and convincing evidence has been advanced by Shannon that this is attributable to the tubular excretion of creatinine. The endogenous creatinine clearance measured by the enzymatic method of Miller and Dubos is identical with the inulin clearance in some instances, but in others it substantially exceeds the inulin clearance, a circumstance consonant with the behavior of exogenous creatinine (21). The apparent clearance of endogenous chromogenic substance, either with or without correction, has been recommended as a measure of glomerular filtration, but such endogenous clearances cannot in our opinion be accepted for this purpose (vide infra).

In view of the theoretical and practical importance of this problem, we have continued our efforts to obtain supplementary evidence on the mechanism of excretion of inulin in the human kidney by searching for additional substances which fulfil the specifications for glomerular excretion without tubular participation. In this search we have been guided generally by the facts that all electrolytes, the excretion of which has been examined, undergo either tubular excretion (phenol red, hippuran, diodrast, iopax, neoiopax) or tubular reabsorption (sodium, potassium, chloride, nitrate, thiocyanate, sulfate, uric acid, etc.). Though this circumstance does not negate the possibility that an electrolyte of suitable properties might escape both tubular excretion and reabsorption, any search in this direction must, at the present time, go forward empirically. We have therefore confined our investigation for the most part to compounds related to the carbohydrates, since no one of the simple carbohydrates, at least, is known to be excreted by the tubules in any species (37), while the process of tubular reabsorption in this group is apparently related to spatial configuration. The present report deals chiefly with the excretion of certain of the hexitols and their derivatives, with supplementary data on the excretion of endogenous creatinine-like chromogens.

Results

Table I summarizes our observations on the simultaneous clearances of the hexitols and their anhydrides in the dog. The agreement of the simultaneous clearances of sorbitol, mannitol, dulcitol, and sorbitan with the creatinine or inulin clearance is such as to leave no doubt that the three hexitols and the first anhydride, sorbitan, on the one hand, and creatinine and inulin, on the other, are excreted by identical mechanisms.

The marked discrepancy between the creatinine clearance and the simultaneous clearances of isomannide and sorbide equally leaves no doubt, since these substances are completely filtrable from the plasma, that about half of the filtered material is reabsorbed from the glomerular filtrate by the tubules. That this reabsorption does not involve the tubular mechanism for the reabsorption of glucose is indicated by the fact that elevation of the plasma glucose to levels at which this mechanism is saturated (32)

TABLE I

Renal Clearances of Hexitols in Dogs

Subject	Date	No. of periods	Hexitol plasma range	Hexitol clearance	Hexitol clearance / Creatinine clearance		Hexitol clearance / Inulin clearance	
					Range	Average	Range	Average
Sorbitol								
			mg. per cent	*cc. per min.*				
Red	3- 1-39	6	150–200	77	0.91–0.99	0.96	0.96–1.03	0.99
Vicky	3-10-39	4	80–150	74	0.93–0.99	0.97	0.93–0.97	0.95
Average						0.97		0.97
Mannitol								
Vicky	3- 7-39	6	219–428	67	0.96–1.00	0.98	0.94–1.00	0.98
Red	3-15-39	4	234–263	60	0.91–0.94	0.92	0.91–0.96	0.94
		4	679–795	59	0.91–0.92	0.92	0.96–0.98	0.97
Average						0.94		0.96
Dulcitol								
Vicky	6- 5-39	5	229–300	55	0.96–1.00	0.98		
	12-11-39	5	257–261	63	0.95–1.00	0.97		
Clemi	6-13-39	6	116–126	72	0.97–1.01	0.98		
Average						0.98		
Sorbitan								
Clemi	6-22-39	6	159–173	52	0.92–1.11	1.02		
Vicky	6-26-39	6	186–230	53	0.97–1.04	0.99		
Average						1.00		
Isomannide								
Vicky	7- 6-39	6	126–175	31	0.43–0.48	0.45		
	11-20-39	5	142–200	31	0.45–0.49	0.47		
Red	7-11-39	4	107–147	28	0.39–0.46	0.41		
		3	408–448	32	0.44–0.54	0.48		
		3	555–568*	43	0.54–0.58	0.56		
Average						0.47		
Sorbide								
Red	4- 8-40	5	90–110	63	0.52–0.57	0.55		

* Plasma glucose, 428 to 458 mg. per cent.

TABLE II

Renal Clearances of Hexitols and Derivatives in Man

Subject	Date	Urine protein	No. of periods	Hexitol plasma range	Inulin clearance / Diodrast clearance	Hexitol clearance	Hexitol clearance / Inulin clearance	
							Range	Average
				mg. per cent		*cc. per min.*		
Sorbitol								
H. F.	2-14-39	Eclampsia*	++	7	102-144	0.14	83	0.99-1.06	1.03
"	2-27-39	" †	-	8	100-112	0.24	100	0.98-1.07	1.03
M. C.	2-21-39	Pre-eclampsia†	-	9	84-107	0.29	96	0.97-1.04	1.00
J. G.	2-24-39	Essential hypertension*	-	7	72-107	0.22	136	0.97-1.04	1.00
M. S.	3- 6-39	Normal†	-	8	104-123	0.17	87	0.92-1.14	1.03
F. P.	6- 8-39	Pre-eclampsia†	-	8	43- 69	0.21	94	0.90-1.04	0.98
Average									1.01
Mannitol								
L. J.	3-22-39	Essential hypertension	-	9	203-316	0.24	79	0.91-1.02	0.98
F. P.	3-28-39	Pre-eclampsia*	+++	8	190-272	0.14	71	0.99-1.03	1.02
"	4- 3-39	" *	+++	8	191-262	0.13	76	0.96-1.10	1.02
M. S.	3-30-39	" *	++	8	212-313	0.18	58	1.01-1.05	1.03
"	5-15-39	" †	-	3	253-256	0.24	65	0.99-0.99	0.99
E. S.	4- 6-39	" †	+	8	152-224	0.24	106	0.93-0.98	0.96
H. L.	11- 3-39	Normal*	-	8	148-201	0.18	120	0.96-1.03	0.99
M. A.	11-27-39	" *	-	8	155-203	0.21	130	0.95-0.99	0.97
Average									0.99

Dulcitol

R. D.	6-21-39	Normal		95-102	0.19‡	122	0.01-0.05	0.04
F. B.	6-28-39	"		131-146		142	0.87-0.03	0.04
A. F.	6-29-39	"		182-205	0.20‡	118	0.80-0.06	0.03
H. U.	12- 7-39	Essential hypertension		107-164	0.20‡	70	0.03-0.08	0.06
	1-18-39	"		93-144	0.20‡	82	0.80-0.09	0.05
V. V.	4-11-40	"		108-146	0.16‡	112	0.92-0.95	0.03
Average								0.04

Sorbitan

C. T.	12-14-39	Essential hypertension		111-155	0.20‡	96	0.93-1.01	0.98
	12-10-39	"		158-209	0.20‡	98	1.05-1.09	1.06
H. U.	1- 9-10	"		163-238	0.20‡	84	0.91-1.06	0.99
Average								1.01

* Antepartum.
† Postpartum.
‡ Not determined simultaneously with hexitol clearance.

does not alter the isomannide-creatinine clearance ratio (see Table I). The fact that raising the plasma level of isomannide itself does not greatly raise this clearance ratio argues against, though it does not definitely disprove, the possibility that the reabsorption of isomannide is an "active" process, in the sense in which the reabsorption of glucose is an active process.

No suggestion can be offered as to why isomannide and sorbide should be reabsorbed by the tubules when sorbitol, mannitol, dulcitol, and sorbitan are not, other than to point out that the first two compounds are second anhydrides, whereas sorbitan is a first anhydride, and the other four compounds are hexahydric alcohols.

Table II summarizes our data on man. As in the dog, the sorbitol, mannitol, and sorbitan clearances are clearly identical with the inulin clearance. The dulcitol clearance in man is consistently slightly below the inulin clearance; whether this is attributable to some systematic analytical error or to a species difference we have been unable to determine, but in view of the identity of these clearances in the dog (Table I), we discount the latter interpretation.

The excretion of isomannide and sorbide in man was not examined, since suitably pure material was not available.

DISCUSSION

The problem of measuring accurately the rate of glomerular filtration in man has assumed increased importance as the possibilities of evaluating tubular activity have evolved (14, 27, 29, 31, 32, 37, 38). The only convincing method of demonstrating the processes of tubular excretion or reabsorption is by the comparison of simultaneous urine-plasma ratios in individual nephrons or, in the case of the mammalian kidney, by comparison of the over-all clearances. Evidence obtained by examining the effects of changing the plasma concentration of a single substance on the absolute rate of its excretion, though capable of revealing tubular excretion or reabsorption when such evidence is positive, cannot when it is negative add more than inferential support to the evidence obtained by the comparative method.

The earlier comparative data on renal clearances in man has been reviewed elsewhere (37). Since that review, only one new line of evidence of this nature has become available: Shannon and

Fisher (32) have shown that during hyperglycemia in the dog, when the glucose reabsorptive mechanism of the tubules is saturated, the clearance of xylose (which is normally reabsorbed to a slight extent) is identical with the creatinine clearance. In unpublished studies, Shannon and Ranges have now shown this to be equally true in man. In the matter of absolute rates of excretion, Miller, Alving, and Rubin (20), using the micromethod (1), have shown that the inulin clearance in any one subject is independent of the plasma concentration of inulin at levels ranging from 5 to 85 mg. per cent, supplementing the demonstration by Shannon and Smith (34) of constant clearance between 50 and 400 mg. per cent.

The present demonstration that the clearances of sorbitol, mannitol, dulcitol, and sorbitan are identical with the creatinine (and therefore inulin) clearance in the normal dog supplements the already abundant evidence that the creatinine and inulin clearances are at the level of glomerular filtration in this animal. Because of analytical difficulties, the clearances of several substances cannot be determined simultaneously, but since we may reasonably equate the clearances of substances which have been separately compared with either the creatinine or inulin clearance we may say that the inulin, creatinine, sorbitol, mannitol, dulcitol, sorbitan, and xylose clearances (the last named during hyperglycemia only) are identical in this animal.[1]

The conclusion that the inulin clearance is at the level of glomerular filtration in man has hitherto rested upon (a) absence of evidence indicating tubular excretion, i.e. absence of any change in the inulin clearance at widely varying plasma levels (5 to 400 mg. per cent); (b) positive evidence of tubular excretion of creatinine in man, consisting of the depression of the creatinine

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[1] The ferrocyanide clearance should be included in this list, though the significance of this clearance, first studied by Van Slyke, Hiller, and Miller (45), remains subject to some doubt, since Miller and Winkler (23) have found that ferrocyanide is reabsorbed in man. On this point Miller and Winkler remark, "We are inclined to interpret our results as representing a species difference between dog and man. It should, however, be pointed out that the levels of serum ferrocyanide in our human experiments are distinctly lower than those in the dog experiments of Van Slyke, Hiller, and Miller, and that in this respect the clearances obtained in their experiments were obtained under conditions differing from ours."

clearance and of the creatinine-inulin clearance ratio on elevation of the plasma creatinine concentration; and (c) the identity of the inulin and creatinine clearances in phlorhizinized man (20, 30, 33, 34). To this evidence is now added the demonstration that the clearances of inulin, sorbitol, mannitol, and sorbitan are identical, while that of dulcitol is so slightly below the inulin clearance that the discrepancy may be due to systematic error. That this identity is a consequence of the identical reabsorption of all these substances is highly improbable.[2]

It is of interest to note that the three hexitols, though excreted alike by the kidney, are metabolized to markedly different degrees. Todd, Myers, and West (44) conclude that sorbitol increases the blood glucose in the dog, and Carr and Forman (2) find that it increases the liver glycogen in the rat. According to Todd et al. (44) less than 50 per cent of this hexitol is excreted in the urine in 24 hours after intravenous injection. Considerable metabolism is also indicated in Waters' report (46). We find that after the intravenous administration of 9 gm. of sorbitol and 8 gm. of inulin in normal man, only 32 per cent of the sorbitol appeared in the urine, whereas 98 per cent of the inulin was excreted.

Dulcitol, on the other hand, does not elevate blood glucose or the R.Q. (3) and it is reported by Ishihara, Kimura, Miyaja, Shentaku, and Sugiyama (15) not to increase liver glycogen in the rat, while Carr and Krantz (3) state that it reduces tissue glycogen and only slightly elevates liver glycogen. In a single observation on a normal man receiving 4 gm. of dulcitol intravenously, we found 8? per cent to be excreted in the urine in 10 hours. Apparently the metabolism of this substance is slight.

Also slight, according to the available evidence, is the metabolism of mannitol. Carr, Musser, Schmidt, and Krantz (5) report that mannitol does not increase the R.Q. of fasting rats, although it increases the blood sugar in rabbits; according to Todd, Myers, and West (44) it does not increase the blood sugar in dogs, and these authors find, in accord with Silberman and Lewis (35), that when it is given per os there is but slight conversion to liver glycogen, although such conversion is evident on long feeding according to Carr and Krantz (4). In two observations in which

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[2] Since this paper was completed, Steinitz (41) has reported the identity of the sucrose and inulin clearances in man.

10 gm. of mannitol and 5 gm. of inulin were given intravenously to normal men, we were able to recover from urine collected during 10.5 hours following the injection 81 per cent of the administered mannitol and 95 per cent of the inulin in one case, and in the other, 89 per cent of the mannitol and 97 per cent of the inulin.

Isomannide is apparently not metabolized to any great extent (17, 16).

It may be noted that no toxic symptoms were apparent in these patients after they had been given as much as 80 gm. of sorbitol or mannitol, 50 mg. of dulcitol, or 70 gm. of sorbitan by intravenous administration over a period of 2 hours.

Clearances of Endogenous Substances Giving the Jaffe Reaction

The apparent creatinine clearance based upon the endogenous substance or substances which yield color with alkaline picrate has been recommended as a measure of glomerular filtration by Popper, Mandel, and Mayer (26), Popper and Mandel (25), Findley (10), and Steinitz and Türkand (43). We have consequently included here observations on the apparent clearance of these endogenous chromogens in man, as determined simultaneously with the inulin clearance.

Popper, Mandel, and Mayer (26) recommend precipitating plasma proteins by picric acid, since this reduces the chromogen content of the filtrate, and we have compared their method with the $BaCO_3-Fe_2(SO_4)_3$ method of Steiner, Urban, and West (40). The first method, as applied by us, consists of the drop by drop addition of 4 cc. of plasma to 12 cc. of saturated picric acid; the mixture is heated in a boiling water bath for 12 to 15 seconds and filtered through Schleicher and Schüll No. 597 filter paper. 10 cc. of cooled filtrate are transferred to an Evelyn colorimeter tube and alkalinized with 0.5 cc. of 10 per cent NaOH. The light absorption is determined after 20 minutes with a No. 520 filter, and the chromogen content read from a standard curve prepared as in the analysis of urine. Urine is diluted to approximately a urine-plasma ratio of 1.0 and 3 cc. of the diluted solution are added to 9 cc. of saturated picric acid solution in the colorimeter tube; 0.6 cc. of 10 per cent NaOH is added and light absorption is read after 10 to 20 minutes.

In the Steiner, Urban, and West method, 4 cc. of 17 per cent

$Fe_2(SO_4)_3$ are added to a mixture of 4 cc. of plasma and 32 cc. of water contained in a 125 cc. Erlenmeyer flask. 6 gm. of precipitated $BaCO_3$ are added[3] and the flask is stoppered and shaken until all evolution of CO_2 has stopped. The mixture is centrifuged and filtered through washed cotton or filter paper, and 4 drops of saturated Na_2SO_4 are added. After the mixture has stood for 15 minutes, the $BaSO_4$ is centrifuged out and the fluid is filtered again. 10 cc. of this filtrate are transferred to an Evelyn colorimeter tube and 5 cc. of alkaline picrate (5 volumes of saturated picric acid plus 1 volume of 10 per cent NaOH) are added. Light absorption is read with a No. 520 filter exactly 10 minutes later. Urines are diluted to approximately the same concentration as the plasma filtrates and analyzed as above without precipitation. Standard curves are prepared with 10 cc. of aqueous creatinine solution handled in the same manner as the urines.

We find that both of the above methods give quantitative recovery of creatinine when this is added to plasma in concentrations of 0.25 mg. per cent or more, if the concentration of the endogenous chromogenic substance as determined by the respective methods is deducted from the total chromogenic substance. A detailed report of these recoveries appears to be superfluous.

The two methods of precipitation, however, give markedly different recoveries of endogenous chromogen from both plasma and urine. In a series of fourteen samples of human plasma, the chromogen present in the picric acid filtrates ranged from 50 to 79.2 (average 66.6) per cent of that present in the iron filtrate. Popper, Mandel, and Mayer (26) report their picric acid filtrates contain about 50 per cent as much chromogen as the Folin picric acid (12), and Ferro-Luzzi and coworkers (8, 9) report that Somogyi's (39) Zn precipitate method yields substantially lower chromogen values than does the Folin and Wu tungstate filtrate (13). Chesley and Chesley (7), using the tungstate filtrate, find that the endogenous chromogen clearance has about the same magnitude as the urea clearance, which fact may be attributed to the high plasma chromogen values given by this filtrate. In urine, although the difference between methods of precipitation is less marked, there is still not identity of behavior; we find in

[3] The $BaCO_3$ should be tested to be certain that, with the quantity of $Fe_2(SO_4)_3$ used, the filtrate will not turn red litmus paper blue.

twenty-one samples that the picric acid filtrate yielded from 85 to 102 (average 92) per cent of the chromogen given by the iron filtrate.

It is clear that these various methods of protein precipitation, at least two of which we have found give 100 per cent recovery of added creatinine, do not yield the same chromogen content in either plasma or urine, and therefore the chromogen in plasma and urine cannot all be creatinine.

Apparent clearances[4] of chromogen in man, as determined by both methods, are given in Table III. These observations have been made during the measurement of the renal plasma flow by the diodrast clearance, when diodrast was present in the plasma to the extent of 0.9 to 1.8 mg. per cent. Diodrast gives no color in the Jaffe reaction, and in our opinion does not influence the apparent clearance of chromogen. The observations have been extended to include high plasma levels of diodrast (20 to 40 mg. per cent), utilized for the measurement of the maximal rate of tubular excretion of diodrast, with a view to determining whether or not "saturation" of the tubules with diodrast affects the apparent chromogen clearance. Inasmuch as the chromogen-inulin clearance ratio invariably falls during the determination of the maximal rate of tubular excretion of diodrast, we infer that part of the urine chromogen is excreted by the tubules.

The data of Table III show that the apparent chromogen clearance as calculated from the picrate filtrate is higher, whether compared with the inulin clearance or with the apparent chromogen clearance as calculated from the iron filtrate. This is to be expected, since the picrate filtrate gives a lower chromogen yield from the plasma than does the iron filtrate.

Popper and Mandel (25) have argued, on the basis of comparison with the xylose clearance, that the apparent chromogen clearance, as determined by them, is at the level of glomerular filtration. Apart from the now demonstrated active tubular reabsorption of xylose (34, 37), the data advanced to support this conclusion are unconvincing, since the chromogen-xylose clearance ratios pre-

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[4] We say apparent clearances, since physiologically the term clearance should be applied only to discrete chemical entities and not to the excretion of unidentified substances.

TABLE III

Renal Clearances of Endogenous Creatinine-Like Chromogens in Man

Subject	Diagnosis	Date	No. of periods	Plasma range	Clearance During control	Clearance During diodrast saturation	Chromogen clearance / Inulin clearance — Picric acid filtrate During control	Picric acid filtrate During diodrast saturation	Iron filtrate During control	Iron filtrate During diodrast saturation
				mg. per cent	cc. per min.	cc. per min.				
H. O.	Normal	1-24-40	6	1.33-1.05	116.9	94.8			0.89	0.78
T. F.	"	1-29-40	8	1.11-1.25	121.7	76.9			0.82	0.84
		1-26-40	8	0.98-1.04	103.2	89.2			0.93	1.04
H. S.	"	2- 2-40	11	0.98-1.02	104.1	119.8			1.08	0.89
		1-31-40	8	0.56-0.67	169.1				1.01	
		2- 5-40	7	0.83-0.91	135.2				0.86	
		3-11-40	4	0.77-0.77	147.3				0.97	
			4	0.61-0.61	185		1.22			
		3-18-40	4	1.02-1.06	121.7		1.18		0.81	
J. B.	"		4	0.61-0.67	178.3					
		2- 7-40	7	0.89-0.93	134.7		1.00			
		3- 6-40	4	0.86-0.86	138.1		1.14			
		3-13-40	4	1.04-1.04	115				1.07	
			4	0.80-0.80	135		1.26			
T. W.	"	2- 9-40	5	0.83-0.86	159.2	144.7	1.01	0.97		
H. U.	Essential hypertension	3- 8-40	6	0.92-0.95	112.5	87.3	1.20	1.09		
		3-15-40	4	1.36-1.40	71.6				0.94	
"	"		4	0.97-0.97	97.1		1.28			
"	"	4- 5-40	6	0.53-0.62	162.6	123.8	1.42	1.18		

sented by Popper and Mandel vary from 0.77 to 4.5, and average 1.35.

The chromogen-inulin clearance ratios reported by Steinitz and Türkand (43) (using the picrate filtrate) for normal subjects average 1.03, but they range from 0.73 to 1.17; in subjects with glomerulonephritis, the range is from 1.04 to 1.73, with an average of 1.37. We have not examined this ratio in glomerulonephritis, but in our normal subjects this ratio is variable and generally exceeds 1.0.

It is clear from the data of Table III and the other investigations cited that the apparent clearance of endogenous chromogenic substance differs markedly when different methods of precipitation of plasma protein are used, and that with no method so far reported is the endogenous chromogen clearance consistently identical with the inulin clearance. The nature of the substances in plasma which participate as chromogens in the Jaffe reaction is still a matter of debate; although creatinine is present among these chromogens (21), the participation of one or more other substances is no doubt the source of variability in the clearance in normal subjects and the even greater variability in subjects with renal disease. Whatever the empirical usefulness of this test, so long as the nature of the substance or substances involved is unknown, its use in the quantitative exploration of renal function, and especially in the evaluation of tubular activity (42), is likely to be a source of considerable error.

SUMMARY

1. Methods for the quantitative determination in the blood and urine of the hexitols, sorbitol, mannitol, and dulcitol; of the monoanhydride, sorbitan; and of the dianhydrides, isomannide and sorbide, are described.

2. The clearances of sorbitol, mannitol, dulcitol, and sorbitan are identical with the simultaneous creatinine or inulin clearances in the dog.

3. The clearances or sorbitol, mannitol, and sorbitan are identical with the simultaneous inulin clearance in man. The dulcitol-inulin clearance ratio averages 0.94; it is believed that the discrepancy between these two clearances in man is possibly attributable to systematic analytical error.

4. Sorbitol and mannitol are cleared from the plasma at the same rate as inulin both when the glomerulus is normal and when it is filtering protein.

5. Isomannide and sorbide undergo tubular reabsorption in the dog; the excretion of these substances in man was not examined.

6. Data are given on the metabolism of the hexitols and their derivatives in man.

7. The data presented here are strong supportive evidence that the inulin clearance is at the level of glomerular filtration in both dog and man.

8. The excretion of endogenous chromogenic substance (Jaffe reaction), as determined in picric acid and iron filtrates of plasma, has been compared with the excretion of inulin in man. The inadequacy of the so called "endogenous creatinine" clearance as a measure of glomerular filtration is discussed.

Methods

The methods of administration of the various substances examined here, and of clearance determination, have been similar to those previously described from this laboratory (6, 14, 38). For administration to dogs the hexitols were used without repurification, but for human administration they were filtered through a Seitz E. K. No. 3 asbestos filter and administered in 7.5 to 10 per cent solution. Heparin is used as an anticoagulant.

Fermentation and Precipitation of Plasma and Urine (Zinc Filtrate)

When the plasma inulin concentration ranges from 10 to 25 mg. per cent, removal of inulin is unnecessary; the hexitol equivalent of inulin is 1.0 and appropriate correction can be made in both plasma and urine. Fermentable sugar must, however, be removed. For this purpose, bakers' yeast is washed six to eight times until the supernatant fluid is clear, and suspended in approximately 20 per cent solution. This solution will keep in the ice box for a week, but must invariably be centrifuged and resuspended on the day of use, and the extracellular water content determined with a hematocrit tube. 2 cc. of plasma or diluted urine are treated with 6 cc. of yeast suspension for 15 minutes at room temperature and then centrifuged. From 2 to 5 cc. of the

supernatant fluid are transferred to a 50 cc. Erlenmeyer flask and, if further dilution is desired, 5 cc. or more of water are added. Precipitation is effected by the addition of 6 cc. of Zn solution and, after thorough mixing, 2 cc. of 0.75 N NaOH. The Zn solution consists of 66.7 gm. of $ZnSO_4 \cdot 7H_2O$ and 167 cc. of 1.0 N H_2SO_4 made up to 2000 cc., and so adjusted with H_2SO_4 or NaOH that 20 cc. require between 7.14 and 7.25 cc. of 0.75 N NaOH to neutralize to phenolphthalein. (If a 1:10 dilution is wanted routinely, the requisite quantity of water may be incorporated in the Zn solution.)   The mixture is well shaken and after 10 minutes transferred to a centrifuge tube, centrifuged, and then filtered through washed cotton.

With high plasma concentrations of inulin, this substance must be converted to fructose and absorbed by yeast before hexitol determination.   10 cc. of the filtrate are hydrolyzed in a calibrated tube by the addition of 1 cc. of 1.0 N H_2SO_4 and heating in a boiling water bath for 15 minutes.   After cooling, 1 drop of 0.02 per cent phenol red solution is added and the sample is neutralized with 1 cc. of 1.0 N KOH, the end-point being carefully adjusted by the drop by drop addition of 1 per cent Na_2CO_3. The mixture is then made up to 15 cc. and 9 cc. of the resulting solution are mixed with 1 cc. of packed yeast and the mixture agitated frequently for 40 minutes.   Fructose is removed by yeast more slowly than is glucose, but if the concentration of inulin in the sample does not exceed 15 mg. per cent, and if plasma and urine samples are adjusted to contain about equal concentrations, no appreciable error results from failure of the yeast to absorb all but slight traces of the fructose or other carbohydrates derived from the hydrolysis of inulin.

Analysis of Sorbitol, Mannitol, and Dulcitol (Periodate Method)

This method is similar to that described by Silberstein, Rappaport, and Reifer (36), and involves the oxidation of glucose-free filtrate with a known excess quantity of KIO_4, and determination of the excess KIO_4 by liberation of I_2 and $Na_2S_2O_3$ titration.   We have found it advantageous to substitute the Somogyi (39) $ZnSO_4$-H_2SO_4 filtrate, as described above, for the Na_2SiO_3 of Silberstein *et al.* and to carry out the liberation and titration of I_2 in an acid rather than neutral solution.

A KIO_4 solution is prepared by adding 2 parts of 5 per cent H_2SO_4 to 3 parts of 0.1 per cent KIO_4. Duplicate samples consisting of 2 cc. of filtrate are pipetted into Pyrex ignition tubes (24 × 200 mm.) and exactly 5 cc. of the acid KIO_4 solution are added to each tube. The tubes are closed with glass tears to effect condensation and are placed in boiling water for exactly 20 minutes. During this time the hexitol, if its concentration does not exceed 20 mg. per cent, will be completely oxidized to formic acid and formaldehyde (11, 18). After cooling, about 0.5 gm. of KI crystals is added to the samples one at a time and the liberated iodine titrated with 0.005 N $Na_2S_2O_3$, 1 drop of a 1 per cent soluble starch solution being added near the end-point. The $Na_2S_2O_3$ is standardized against 0.001 N KIO_3.

Two dummy samples of the KIO_4 solution, with 2 cc. of water substituted for the filtrate, are boiled simultaneously with each group of unknowns and titrated to obtain the standard KIO_4 titer. Since the KIO_4-KI mixture is titrated in acid solution, all the iodine liberated by the unreduced KIO_4 is titrated with $Na_2S_2O_3$, and consequently in the analysis of the unknowns the $Na_2S_2O_3$ equivalent of the dummy KIO_4 is diminished by slightly less than one-quarter (for example from 19.5 to 15.7 cc.). In view of this fact, and to afford maximal accuracy in titration, we have utilized a special burette of the Bang type, the upper portion consisting of a bulb graduated to deliver 15 cc. between two marks, the lower portion consisting of a 5 cc. burette 15 inches in length and graduated in 0.02 cc. Duplicate samples should check within 0.06 cc.[5]

The difference between the standard KIO_4 titer and the titer of the unknown, multiplied by 9.2/2, and by the appropriate dilution factor, gives the mg. per cent of hexitol in the original sample. (The figure 9.2 is a conversion factor which takes into account the incomplete oxidation of the hexitol and is applicable to sorbitol, mannitol, and dulcitol.)

[5] In approximately neutral solution, KIO_4 liberates only 1.0 equivalent of iodine, and Silberstein et al. (36) recommend that the titration of the KIO_4 + KI mixture be carried out in neutral solution. This has the advantage of eliminating the residual titration figure of 15 cc., as described above, but we find the adjustment of the mixture to neutrality difficult and uncertain, and prefer to titrate the total KIO_4 iodine in acid solution. The use of a special burette, as described above, circumvents the single disadvantage of this procedure.

The plasma "hexitol" blank (about 10 mg. per cent) must be determined by analysis of a control sample of plasma as described above. When the urine is treated with yeast before precipitation, the blank is negligible. A small quantity of hexitol is lost on the yeast, but in so far as this loss is uniform in blood and urine samples when these are handled alike, it introduces no error in the calculations of the clearances. Creatinine does not contribute to the blank.

In a series of recoveries of sorbitol at 50 to 200 mg. per cent from sixteen samples of dog and human plasma the average deviation in recovery was 1.6 mg. per cent, with a maximal deviation of 4.6 mg. per cent. Four of these samples contained 30 to 60 mg. per cent creatinine, which did not increase the error of recovery.

Sorbitol and mannitol do not penetrate the red blood cells of human blood *in vitro*, as shown by the fact that 95 to 96 per cent of added hexitol is recoverable from plasma 2, 15, and 33 minutes after the addition of known quantities to whole blood.[6] A small apparent loss by this method is explicable from failure to obtain a plasma-free hematocrit. The *in vitro* penetration of the other compounds was not examined.

Neither sorbitol, mannitol, isomannide, nor sorbide is bound by human plasma proteins, as shown by ultrafiltration and dialysis as described by Shannon (29). Allowing for a protein concentration of 6 gm. per 100 cc., and deducting the blank as determined in a control ultrafiltrate from the same plasma, we recovered 98 to 100 per cent of the hexitols from the ultrafiltrate at a concentration of 125 to 150 mg. per cent. The other compounds were not examined.

Analysis of Sorbitan, Isomannide, and Sorbide (Ceric Sulfate Method)[7]

Plasma and urine are precipitated and treated with yeast as in the periodate method described above. The oxidizing agent is

[6] This is also true of diodrast (38), but White and his coworkers (47, 48) have shown that to some extent this substance penetrates the red blood cells of both dog and man *in vivo*. A plausible explanation of this fact is that during the circulation of the blood through the capillary bed the permeability of the red cells is increased by distortion.

[7] A similar method in which ceric sulfate is utilized for the determination of isomannide has been used by Krantz and Carr (16), the details of

prepared according to the description of Miller and Van Slyke (22).   10 cc. of 0.1377 N ceric sulfate are diluted to 100 cc. with 5 per cent H_2SO_4.   2 cc. of filtrate are pipetted into tubes, 5 cc. of the ceric sulfate solution are added, and the tubes are closed with tears and placed in a boiling water bath for 30 minutes. Two dummy samples are boiled with each group of unknowns. After cooling, 0.5 gm. of KI is added to each tube and the liberated I_2 is titrated with 0.005 N $Na_2S_2O_3$, with an ordinary burette delivering 15 cc.   Standard curves, prepared by the analysis of aqueous solutions of sorbitan, etc., in the above manner, are used for the conversion of cc. of ceric sulfate to concentration of the hexitol derivative.   Such curves are nearly straight lines and can be extrapolated to zero ordinates.   Plasma and urine blanks, which are considerably higher than in the KIO_4 method, are determined on control samples, the latter being calculated as mg. per minute and deducted from all experimental periods.   The sorbitan equivalent of inulin is 1.0.

Inulin determinations were made on the above Zn filtrates either by the method of Smith *et al.* (38), or by the micromethod of Alving, Rubin, and Miller ((1) and personal communication). The determination of *creatinine* is described in the body of the paper.   *Diodrast iodine* was determined by the method described by Smith *et al.* (38).

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THE EXCRETION OF HOMOGENTISIC ACID AND OTHER TYROSINE METABOLITES BY THE VITAMIN C-DEFICIENT GUINEA PIG*

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In the course of the experiments demonstrating that the metabolism of the melanin pigment precursors, tyrosine and dihydroxyphenylalanine, increases the vitamin C requirement of the guinea pig (1) it was learned that those animals receiving tyrosine excreted homogentisic acid in the urine. Furthermore, the amount of the metabolite appeared to be inversely proportional to the amount of ascorbic acid received. Investigation of this point by Sealock and Silberstein (2) has shown that the production of the experimental alkaptonuria is dependent upon a deficiency of the vitamin and that when this deficiency is removed the homogentisic acid no longer appears. On identification of this substance by isolation of the dibenzoylhomogentisamide other tyrosine metabolites were found in the urine of the vitamin C-deficient animals. With the aid of the 2,4-dinitrophenylhydrazine reagent of Penrose and Quastel (3) the corresponding keto acid, *p*-hydroxyphenylpyruvic acid, was identified. The α-hydroxy derivative also has been shown to be present but in smaller amount than in the case of the keto acid. Of even greater significance was the finding that these metabolites as in the case of the homogentisic acid are no longer excreted when an adequate amount of ascorbic acid is administered to the guinea pigs.

While these experiments were in progress, there appeared a paper by Levine, Marples, and Gordon (4) reporting that premature infants receiving cow's milk as the source of protein excreted the keto and hydroxy acid derivatives of phenylalanine and tyrosine, and that the excretion of the metabolites was completely prevented within 48 to 72 hours by the administration of vitamin C. These independent findings furnish additional evidence of the relation of ascorbic acid to the metabolism of the aromatic amino acids and emphasize the importance of a complete analysis of this action of the vitamin.

In the meantime, it is the purpose of this paper to report in greater detail further experiments in connection with the effect of ascorbic acid upon the excretion of homogentisic acid, and our findings with regard to the keto and hydroxy acids.

<div align="center">EXPERIMENTAL</div>

Male guinea pigs of approximately 300 gm. were housed in individual metabolism cages and the urine was collected in 24 hour samples, a small amount of mineral oil being used in the collecting flasks and sufficient hydrochloric acid to maintain the samples acid to Congo red at all times. Immediately after collection the filtered urine was analyzed for homogentisic acid by the method of Briggs (5), for keto acid by the method of Penrose and Quastel (3), and for the total tyrosine value by the method of Folin and Ciocalteu (6) as modified by Medes (7). Since p-hydroxyphenylpyruvic acid also reduces the phosphomolybdate reagent of Briggs and at a different rate (7), the homogentisic acid values were obtained by reading 5 minutes after the addition of the final reagent and then deducting the keto acid present. The total tyrosine value was also corrected for the amount of the keto acid present and thus represents p-hydroxyphenyllactic acid and tyrosine. On occasion the lactic acid derivative was determined by extraction of the acidified urine with ether and again determining the total tyrosine and keto acid values. As a further check on the specificity of these analytical procedures the presence of each of the indicated compounds has been established by isolation and identification in the usual fashion.

The basal diet consisted of ground Purina Rabbit Chow (complete ration) which had been exposed to air in thin layers to remove

the traces of vitamin C originally present. This diet was then supplemented with 0.5 cc. of cod liver oil weekly and 0.9 gm. of brewers' yeast, Harris, daily. This relatively large amount of yeast insures an adequacy of vitamin B_1, a particularly important point since Closs and Fölling (8) have demonstrated the positive effect of this factor in preventing the excretion of phenylpyruvic acid. The l-tyrosine was an analytically pure sample isolated from protein hydrolysates by the customary procedure. The daily amino acid supplement was fed by mixing the required amount in a portion of the basal diet that was readily consumed
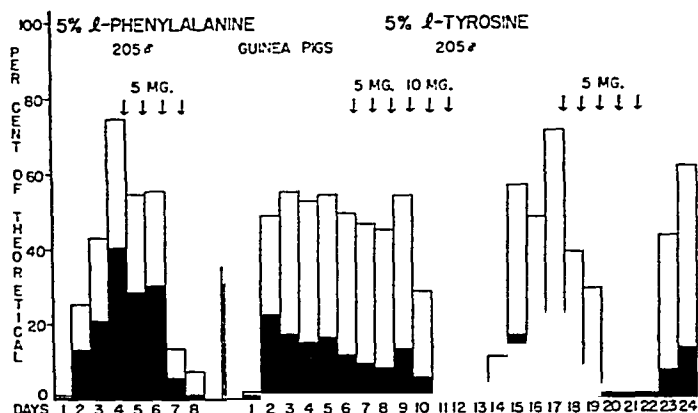
FIG. 1. Homogentisic acid (solid columns) and keto acid (clear columns) excretion. The administration of ascorbic acid, in the amounts indicated, is shown by arrows. The per cent of theoretical is calculated from the amount of supplement eaten.

or by the feeding of a weighed amount of the diet containing 5 or 10 per cent of the amino acid. Ascorbic acid was administered as desired by pipetting the fresh solution in redistilled water directly into the mouth of the animal.

The homogentisic acid and the phenylpyruvic acid excretion resulting from the feeding of l-tyrosine and l-phenylalanine to guinea pigs receiving no vitamin C and the prompt removal of these compounds from the urine with the subsequent administration of the vitamin are illustrated in Fig. 1, in which the level of metabolite is plotted as the per cent of the theoretical, based on

the amount of supplement eaten.   Of eleven animals receiving 0.5 gm. of tyrosine daily, each exhibited similar excretion values, and of these, six, with an intake of 5.0 mg. of ascorbic acid per day, within 48 hours excreted urine containing only traces of the three compounds.   Four were given doses of 10 mg. with similar results.   With the remaining animal, a dose of 1 mg. proved effective in 72 hours.   The feeding of higher levels of the amino acid furnished essentially similar excretion values, as shown in Table I, which also includes the daily amount of ascorbic acid which in spite of the continued high ingestion of the precursor

TABLE I

Tyrosine Metabolite Excretion on Vitamin C-Deficient Diet

Guinea pig No.	Tyrosine		Average daily urinary values*				Ascorbic acid†
	Level	Daily consumption	Keto acid	Homogentisic acid	Tyrosine-lactic acid	Total	Daily dose
	per cent	mg.	per cent	per cent	per cent	per cent	mg.
201	5	680	29.3	11.5	21.3	62.1	5.0
202	5	620	39.7	21.1	16.8	77.6	5.0
205	5	680	38.5	14.6	25.8	78.9	10.0
213	5	756	31.7	9.5	18.8	60.0	5.0
213	5	983	30.4	9.4			10.0
203	10	840	23:8	9.7	15.3	48.8	5.0

* Per cent of theoretical calculated from amount of supplementary tyrosine eaten.

† Amount of ascorbic acid daily, which prevented metabolite excretion within 48 to 72 hours.

effected the removal of the metabolites within 48 to 72 hours.   Of the tyrosine-lactic acid values 6 to 10 per cent is tyrosine, an amount which is also present in the urine during the vitamin feeding period, and in fact represents the only substance relative to these experiments which has been identified in the urines of the vitamin periods.   The remainder consists of the lactic acid derivative.   These values are tabulated together, since the ether fractionation and subsequent analyses were not always carried out.

That the efficacy of a given dose of the vitamin is dependent on the state of vitamin C saturation of the tissues is shown in Fig. 2. In the one case a dose of 0.5 mg. was sufficient to prevent the

excretion of the compounds, but with the appearance of symptoms of scurvy the metabolites gradually increased in concentration and again disappeared with a larger dose of the vitamin. In the second case the guinea pig was definitely scorbutic when the 0.5 mg. doses were begun and failed to respond, but responded promptly to the higher doses. In all cases it has been apparent that the animals exhibiting the more prominent symptoms have required the greater amount of vitamin to remove completely the metabolites from the urine. This finding is in agreement with
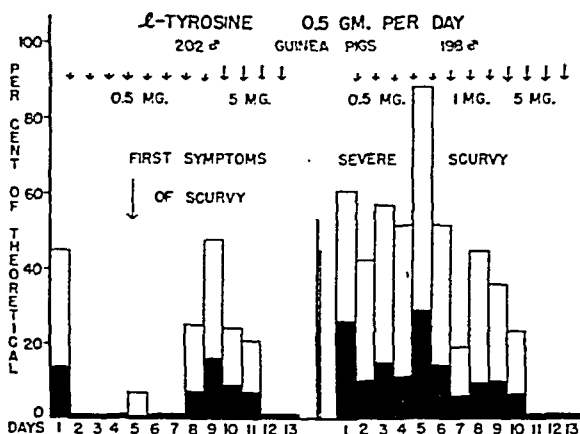
FIG. 2. Metabolite excretion and vitamin C saturation. Homogentisic acid, solid columns; *p*-hydroxyphenylpyruvic acid, clear columns; ascorbic acid, arrows.

those of Levine and his coworkers who point out the importance of the degree of saturation of the tissues with vitamin C.

With the demonstration that ascorbic acid will prevent the metabolite excretion, the question arises as to the specificity of the vitamin in this relationship. It becomes necessary to prove that it is a result inherent in the vitamin molecule rather than due to a non-specific action of a reducing substance. To test these possibilities, we have first used *d*-isoascorbic acid which differs from the natural substance only by the spatial configuration of 1 carbon atom and possesses an antiscorbutic activity of one-twentieth to one-fiftieth (9, 10) that of *l*-ascorbic acid. As shown in Fig. 3, 10 mg. of the isomer, an amount equal to the dose of

l-ascorbic acid which has always prevented the excretion of the metabolites, was without effect even though administered over a period of several days. Furthermore, it is apparent that after the successful use of the vitamin the return of 10 mg. of *d*-iso-ascorbic acid to the régime fails to prevent the reappearance of the metabolites in the urine.

When to one of these animals 20 times the dose of the isoascorbic acid or 200 mg. was given, an effect identical to that produced with 10 mg. of the natural vitamin was observed within 72 hours.
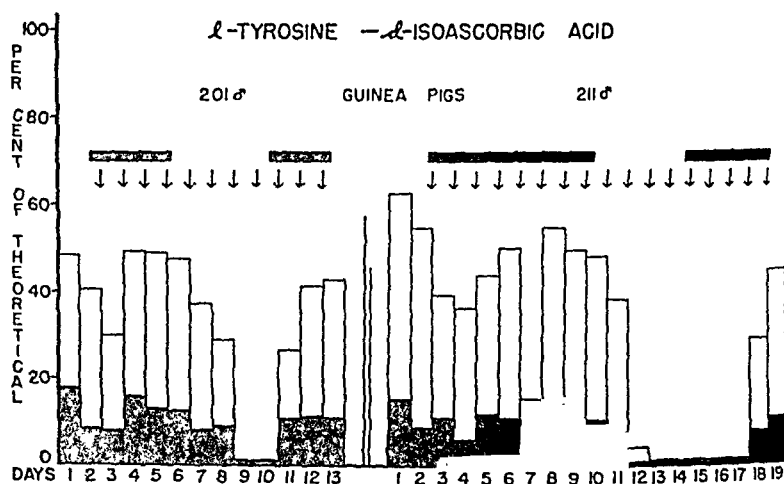
FIG. 3. Metabolite excretion and *d*-isoascorbic acid on a 5 per cent tyrosine diet. Homogentisic acid, solid columns; *p*-hydroxyphenylpyruvic acid, clear columns; 10 mg. of *l*-ascorbic acid, arrows; 10 mg. of *d*-iso-ascorbic acid, arrows with a bar above.

Since phenylalanine is also a precursor of homogentisic acid, it has been fed under similar conditions, with results entirely analogous to those obtained with tyrosine, as is shown in Fig. 1. While other amino acids would not be expected to give rise to homogentisic acid, the relation of ascorbic acid to the production and excretion of the corresponding keto acid may be tested. For this purpose tryptophane and glutamic acid were fed to vitamin-deficient guinea pigs in 0.5 gm. amounts daily. Even though the animals were extremely deficient as evidenced by marked symptoms of scurvy, no keto acid appeared in the urine. This need not be considered evidence of a separate pathway for

the conversion of tyrosine and phenylalanine to their respective keto acids, for the possibility remains that the carbon residue in the case of tryptophane and glutamic acid may be more readily oxidized or perhaps converted to substances not determined by the analytical procedures employed. These latter possibilities have not yet been investigated.

DISCUSSION

The results reported earlier by us (2), as well as those by Levine and his coworkers (4), and in the present paper clearly indicate that the metabolic handling of phenylalanine and tyrosine is dependent upon the presence in the animal body of an adequate amount of vitamin C. These findings formulate a new rôle for ascorbic acid; namely, that of participating either directly or indirectly in protein metabolism. This view-point is further justified by the experiments with d-isoascorbic acid, which indicate that the prevention of metabolite excretion is inherent in the antiscorbutic activity of the molecule. Additional experiments designed to test this possibility more closely as well as experiments relating to the mode of operation of the vitamin are in progress.

The excretion of homogentisic acid by the guinea pig and lack of excretion of this substance by the premature infant constitute the one essential difference between these experiments and those of Levine, Marples, and Gordon (4). Since Papageorge and Lewis (11) found an intake of 300 mg. of phenylalanine per 100 gm. of rat per day necessary to produce alkaptonuria in this species, it is conceivable that the production and excretion of homogentisic acid are dependent upon a minimum intake of the precursors. On the other hand, it seems logical that an animal incapable of synthesizing the vitamin would require less supplement to produce the anomaly. Our own experiments offer evidence to support this point, if one may compare the rat and guinea pig on a weight basis, in that the daily amount of supplement eaten has varied from 150 to 330 mg. per 100 gm. of animal, with homogentisic acid excretion resulting in each instance.

Since extra vitamin C has proved entirely ineffective in preventing the excretion of homogentisic acid by an alkaptonuric patient (12–14), the metabolism of this compound in the normal individual is probably accomplished by several different enzyme factors.

Furthermore, we have suggested that the vitamin is not the missing one in the hereditary condition, but rather some other factor as yet unknown.

SUMMARY

The feeding of tyrosine in amounts of 0.5 gm. or more daily to guinea pigs on a vitamin C-deficient diet results in the excretion of homogentisic, *p*-hydroxyphenylpyruvic, and *p*-hydroxyphenyllactic acids. The administration of *l*-ascorbic acid in amounts of 10 mg. or less daily completely prevents the excretion of these metabolites.

Evidence is presented to indicate the importance of the degree of vitamin C saturation in the tissues in producing or preventing the anomaly.

The specificity of the ascorbic acid action is demonstrated by the fact that 10 mg. of *d*-isoascorbic acid are ineffective in replacing a similar weight of *l*-ascorbic acid, but 200 mg. of the former, an amount equivalent to the natural vitamin in antiscorbutic activity, are able to prevent the metabolite excretion.

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THE NITROGEN, COPPER, AND HEMOCYANIN CONTENT OF THE SERA OF SEVERAL ARTHROPODS[*]

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In spite of several investigations on the hemocyanin and other protein content of invertebrate blood sera (Redfield, 1934; Roche, 1936, and papers quoted therein) there is still some uncertainty concerning (1) the amount of hemocyanin in the sera of different species and individuals, (2) the presence of other proteins besides hemocyanin, and (3) the copper to nitrogen ratios in purified hemocyanins. In making new analyses for electrolytes in several kinds of invertebrate sera (Cole, 1940), it seemed desirable to secure further information on those questions from the sera of *Homarus americanus*,[1] *Cancer borealis*,[1] and *Limulus polyphemus*.[1]

Materials and Methods

All of the animals used were in excellent condition as far as could be determined, and had been living in the environment from which they were removed for long periods of time. They were bled immediately upon removal from sea water. The blood was vigorously stirred for at least 10 minutes to insure complete clotting and the serum was filtered off through rapid paper. Since it is well known that clotting of the bloods involves clumping of the blood cells and coagulation of proteins released from those cells, it is extremely important to remove all the cells

[*] The work reported here was done under the sponsorship of the Bureau of Biological Research, Rutgers University, and with the assistance of General Scientific Project, Division of Professional and Service Projects, Work Projects Administration of New Jersey.

[1] The specimens of *Homarus* and *Cancer* were collected from Frenchman's Bay near Salsbury Cove, Maine; those of *Limulus* from Pierces, New Jersey, on Delaware Bay.

and the clot from the serum before analyses are made. If the sera were not analyzed immediately, 1 ml. of toluene was added to each 100 ml. of serum as a preservative. No evidence was obtained that the toluene in any way caused errors in analysis. Some of the clots were analyzed separately to determine their copper and nitrogen content.

Total nitrogen and non-protein nitrogen of the sera were determined by the Kjeldahl method with the digestion mixture of Folin and Wright (1919). In order to minimize contraction of the solution caused by removal of the protein from sera, the following method of precipitation was used. 10 ml. of serum were mixed with 15 ml. of distilled water, following which 10 ml. of 20 per cent trichloroacetic acid were added drop by drop with constant stirring. After the mixture had stood until the supernatant fluid became clear, the precipitated protein was removed by centrifugation and aliquots of the supernatant were taken for analyses. Total nitrogen of the purified hemocyanin was determined by the Pregl micro-Kjeldahl method, with selenium oxychloride as a catalyst.

A microdetermination for copper was used which is based on the method described by Redfield, Coolidge, and Shotts (1928). Since it was found possible to eliminate the electrolytic deposition of copper in this method and to improve on their iodometric determination by utilizing the technique of Foote and Vance (1935, 1936), the procedure will be given in detail. 2 ml. of serum or 0.1 gm. of hemocyanin, 10 ml. of concentrated HNO_3, and 2 ml. of concentrated H_2SO_4 were mixed in a 100 ml. Erlenmeyer flask and digested on a hot-plate until clear. After evaporation to dryness the residue was dissolved in 4 ml. of 0.1 N H_2SO_4 and the solution heated on a water bath for 5 minutes. It was cooled, and buffered to pH 3.8 by adding 2 ml. of 6 N acetic acid and 0.17 ml. of 6 N NH_4OH. 4 ml. of 20 per cent KI solution were added and the iodine was titrated with 0.0015 N $Na_2S_2O_3$, standardized against $CuSO_4 \cdot 5H_2O$ (Foote, 1938), until the iodine color had almost disappeared. 1 ml. of 1 per cent starch was added and the titration continued. Just before the end-point was reached, 2 ml. of 20 per cent NH_4CNS were added and the titration was completed, the blue color being detected last in the meniscus. Deviations between duplicate determinations

should not exceed 1 per cent. This iodometric method was compared with the electrolytic method for the determination of copper in the digest by means of the technique and apparatus described by Clarke and Hermance (1932). The good agreement between the two methods is illustrated by the data in Table I.

The hemocyanins were prepared by isoelectric precipitation and by electrodialysis, as follows: (1) In the *isoelectric precipitation* the serum was dialyzed against distilled water until free of salts, after which the hemocyanins were precipitated by adjusting the solutions to their respective isoelectric points. They were redissolved in a minimum amount of 0.01 N NaOH and reprecipitated by adjusting the solution formed to the isoelectric point

TABLE I

Comparison between Iodometric and Electrolytic Determinations for Copper in Copper Sulfate Solutions and Sera

Sample	No. of determinations	Iodometric method		Electrolytic method	
		Average Cu	Mean deviation	Average Cu	Mean deviation
		γ		γ	
CuSO₄.................	3	404.1	0.2	409.8	4.5
Serum.................	4	379.1	0.6	379.6	7.1
"	2	213.6	0.0	219.6	0.5
"	3	161.5	0.7	162.7	1.8
"	2	57.6	1.9	57.5	0.5
CuSO₄.................	5	46.2	0.3	46.5	0.5

with 0.01 N HCl. This reprecipitation at the isoelectric point was repeated three times. The isoelectric points used were as follows: *Homarus* 4.9, *Cancer* 4.7, and *Limulus* 6.3. The washed hemocyanin was dried to constant weight under reduced pressure at room temperature. Storing over P_2O_5 for 1 week did not alter this weight significantly. (2) *Electrodialysis* was carried out with a technique similar to that described by Dhere (1920). A three compartment dialyzer made of glass was used for this purpose. A platinum electrode was immersed in each of the outer chambers which were kept filled with running distilled water. The serum was placed in the middle chamber which was separated from the outer chambers by collodion membranes.

A direct current of 180 volts was applied to the platinum electrodes and 5 to 10 milliamperes passed through the dialyzer. The hemocyanin was precipitated at the membrane near the anode where the isoelectric point was soon reached. It was necessary to remove nearly all of the salts from the serum by dialysis, before the potential was applied across the electrodes, in order to prevent denaturation of the hemocyanin by electrodialysis. Such denaturation was probably caused by the accumulation of acid around the membrane near the anode.

Results

Analyses for nitrogen and copper in three lots of sera from each species are presented in Table II, and the analyses of the purified hemocyanins from the same species in Table III. By means of the percentages of copper in Table III the gm. of hemocyanin per liter in each lot of serum were calculated (last column, Table II). When the clots were analyzed separately, only traces of copper were found, proving that no hemocyanin had been lost in the clotting process.

It is apparent that large differences occur in the nitrogen and copper content of sera from different groups of individuals in each species. The copper to protein nitrogen ratio, however, was constant in *Limulus* sera, and nearly so in *Homarus* and *Cancer* sera, which means that the amounts of protein (hemocyanin) vary considerably (from 4.6 to 9.8 per cent in *Limulus*, from 1.7 to 3.1 per cent in *Homarus*, and from 1.5 to 3.0 per cent in *Cancer*).

Similar variations are given by Redfield (1934). It is likely that the differences in the concentration of hemocyanin in sera of different individuals and in different species may be associated with differences in the amount of colloidal osmotic pressure needed by the individual or the species and in the respiratory requirements of each.

Since the copper to nitrogen ratios for the purified hemocyanins (Table III) were essentially the same as the copper to protein nitrogen ratios of the sera (Table II), it may be concluded that the only protein in these sera is hemocyanin. The small amounts of other protein in Lots 1 and 2 of *Cancer* sera were probably due to failure to remove all of the clot.

From the close similarity of the copper to nitrogen ratios obtained from the purified hemocyanins of the four species and of the copper to protein nitrogen ratios of the three kinds of sera (except Lots 1 and 2 of *Cancer*) it may be further concluded

TABLE II

Nitrogen and Copper Content and Hemocyanin in Sera of Limulus, Homarus, and Cancer

Lot No.	Total nitrogen	Non-protein nitrogen	Protein nitrogen	Copper	$\frac{Cu}{Protein\ N}$	Hemo-cyanin*
	mм per l.	mм per l.	mм per l.	mм per l.		gm. per l.
1. *Limulus*	560	15.0	545	1.26	0.0023	46.3
2. "	573	18.0	555	1.26	0.0023	46.3
3. "	1185	8.9	1176	2.69	0.0023	98.8
1. *Homarus*	409	23.5	385	0.82	0.0021	31.2
2. "	252	8.2	244	0.55	0.0022	20.9
3. "	214	18.3	196	0.45	0.0023	17.1
1. *Cancer*	480	24.1	456	0.85	0.0019	29.8
2. "	235 ,	20.3	215	0.43	0.0020	15.1
3. "	369	20.4	349	0.84	0.0024	29.5

* Calculated values from data in Table III.

TABLE III

Copper and Total Nitrogen in Purified Hemocyanins Prepared by Isoelectric Precipitation Method, and Copper to Nitrogen Ratios in Hemocyanins Prepared by Electrodialysis

Source of hemocyanins	Isoelectric precipitation			Electrodialysis, Cu:N ratio
	Copper	Total nitrogen	Cu:N ratio	
	per cent	per cent	mм	mм
Limulus polyphemus	0.173	16.10	0.00237	0.00227
Homarus americanus	0.167	16.22	0.00226	0.00213
Cancer borealis	0.181	16.15	0.00246	
Callinectes sapidus				0.00243

that the ratio of copper to protein is essentially the same in these hemocyanins. The differences in the ratios of Table III are considered due to differences in the physicochemical state of the hemocyanin when precipitated from the serum.

DISCUSSION

The percentages of copper in the hemocyanins of *Limulus,* *Homarus,* and *Cancer* reported here are all very close to the values found in the literature reviewed by Redfield (1934) and Roche (1936). The most thorough studies on the copper content of *Limulus* hemocyanin were made by Redfield, Coolidge, and Shotts (1928), Hernler and Philippi (1933), and Roche (1936), whose best average value was 0.173 per cent. For *Homarus* hemocyanin Hernler and Philippi (1933) reported 0.187 per cent, which we believe is at least 0.01 per cent too high. No previous analyses have been found for *Cancer borealis,* but the percentage reported here is within the range reported for other crabs. Roche (1936), for example, found 0.182 per cent in the amorphous form of *Carcinus maenas* hemocyanin.

The percentages of nitrogen in Table III are slightly lower than some of the values for arthropod hemocyanins reported in the literature, the average of which is 16.8 per cent. The copper to nitrogen ratio calculated from the data of Hernler and Philippi (1933) is 0.00225 for *Limulus* and 0.00242 for *Homarus.*

Furthermore the present data confirm the suspicion often expressed in earlier reports that hemocyanin is the only protein in the sera after complete removal of the clot.

SUMMARY

1. An improved method for determining copper in hemocyanin from blood sera of invertebrate animals is described.

2. New data on the copper and nitrogen content of the sera and of the hemocyanins from *Limulus polyphemus, Homarus americanus, Cancer borealis,* and *Callinectes sapidus* are presented

3. The concentration of hemocyanin in those sera varies as much as 100 per cent in different individuals.

4. The ratios of copper to protein in the purified hemocyanin from those sera are all essentially alike.

5. The clotting process does not remove any detectable amount of hemocyanin from the sera.

6. If the clot is completely removed, the sera contain only one protein, the hemocyanin.

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THE EFFECT OF BIOS ON THE NITROGEN METABOLISM OF YEAST

I. AMMONIA AND CARBAMIDE*

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Study of the nitrogen metabolism of yeast has been facilitated by an understanding of the bios requirements of yeast. Synthetic or highly purified bios fractions which do not contain significant quantities of assimilable nitrogen may be employed in an otherwise synthetic solution. In such cases the growth obtained will be directly proportional to the amount of easily assimilated nitrogen present, as in the case of ammonium sulfate. On the other hand, the non-availability of the nitrogen in a substance will be made apparent by the absence of significant growth.

With this kind of technique an examination of various organic nitrogen compounds was undertaken. In the course of this study evidence was found that Bios IIA and Bios IIB may be uniquely connected with nitrogen metabolism. A culture of *Saccharomyces cerevisiae* Type B (requiring Bioses I, IIA, and IIB) gave poor growth on carbamide as compared with ammonium sulfate. Closer investigation disclosed that, in order to obtain a normal crop with carbamide nitrogen, the Bios IIB supplement had to be raised. At the same time it was found that the β-alanine (Bios IIA) supplement could be decreased. These facts suggest that a reexamination will be in order of reports such as Thorne's (1) who found that, "Urea and uric acid are mediocre nutrients of approximately the same value as some of the poorer amino-acids," inasmuch as a sufficient Bios IIB supplement makes urea a good nitrogen source.

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In the present communication we have examined the ammonia-carbamide-bios relationship with eight yeasts, four of Type A and four of Type B, in order to determine how far the conclusions may be generalized. The rôle of bios in a mixture of ammonia and carbamide was also studied. Work in progress indicates that several other nitrogen sources have characteristic bios requirements.

EXPERIMENTAL

Growth tests were performed in the manner previously described (2) and crops are reported on the same basis; *i.e.*, a crop

TABLE I

Yeast Crops on Ammonium and Carbamide Nitrogen As Affected by Bios IIB Increments in Presence of Excess β-Alanine (50 Micrograms)

Yeast strain No.	Ammonium sulfate			Carbamide		
	1 ml. Bios IIB	2 ml. Bios IIB	4 ml. Bios IIB	1 ml. Bios IIB	2 ml. Bios IIB	4 ml. Bios IIB
2335 (Type A).............	40	105	150	20	70	110
Delft I (Type A)..........	55	95	155	30	60	120
Luft II (" ")..........	110	150	190	40	105	180
Rasse XII (Type A).......	95	165	170	60	110	150
4226 (Type B).............	90	130	180	40	85	160
765 (" ").............	50	100	190	25	95	140
4125 (" ").............	100	150	180	40	110	170
Spc. 152 (Type B).........	105	150	185	30	80	170

of 220 indicates the growth of 1 gm. of moist yeast from a 1 mg. inoculum. Ammonium sulfate (150 mg.) and carbamide (100 mg.) were added as indicated. Type A yeasts were supplemented with thiamine and vitamin B_6 in addition to Bios I (inositol), Bios IIA (β-alanine), and Bios IIB. The Bios IIB solution is the same carbon eluate from cane molasses residues previously employed.

Yeast Cultures—Four strains of *Saccharomyces cerevisiae* of both Types A and B were studied.

Type A—Strain 2335, American Type Culture Collection; Strain Delft I, *Centraalbureau voor Schimmelcultures;* Strains *Luft II* and *Rasse XII, Institut für Gärungsgewerbe.*

Results

The growth of a given yeast crop on carbamide requires more Bios IIB than the same crop on ammonium sulfate. All eight yeasts behave alike in this respect (Table I). All samples in Table I were supplemented with 50 γ of β-alanine. The actual crops obtained differ from yeast to yeast, but the trend is the same in all cases.

In Table II 4 ml. of Bios IIB solution were added to each sample. This is sufficient to give crops with all of the yeasts in excess of

TABLE II

Yeast Crops on Ammonium and Carbamide Nitrogen As Affected by β-Alanine Increments in Presence of Excess Bios IIB

Yeast strain No.	Ammonium sulfate			Carbamide		
	0.5 γ β-alanine	1.0 γ β-alanine	2.0 γ β-alanine	0.5 γ β-alanine	1.0 γ β-alanine	2.0 γ β-alanine
2335 (Type A)	10	20	70	50	70	100
Delft I (Type A)	10		45	45	60	90
Luft II (" ")	45	90	130	70	100	120
Rasse XII (Type A)	5	15	30	30	90	130
4226 (Type B)	15	40	120	70	100	130
765 (" ")	40	70	100	60	80	105
4125 (" ")	40	85	140	90	110	150
Spc. 152 (Type B)	80	100	160	70	80	130

150 on ammonia nitrogen, provided sufficient β-alanine is present. However, as the data show, when β-alanine is low the crops are limited; furthermore, the *carbamide crops are higher* than the corresponding ammonia crops. It would therefore appear that under these experimental conditions the β-alanine requirement is greater for assimilation of ammonia nitrogen than for carbamide nitrogen.

With one of the Type B yeasts (Strain 4226) a mixture of ammonia and carbamide was also tried. The results of this series, which included the necessary controls on ammonia and carbamide, are shown graphically in Fig. 1. The first set of curves shows that, when β-alanine is the limiting factor in a mix-

ture of the two nitrogen sources, growth is very poor. The mixture is poorer than either alone.   On the other hand, as the second set of curves shows, when Bios IIB is the limiting factor,
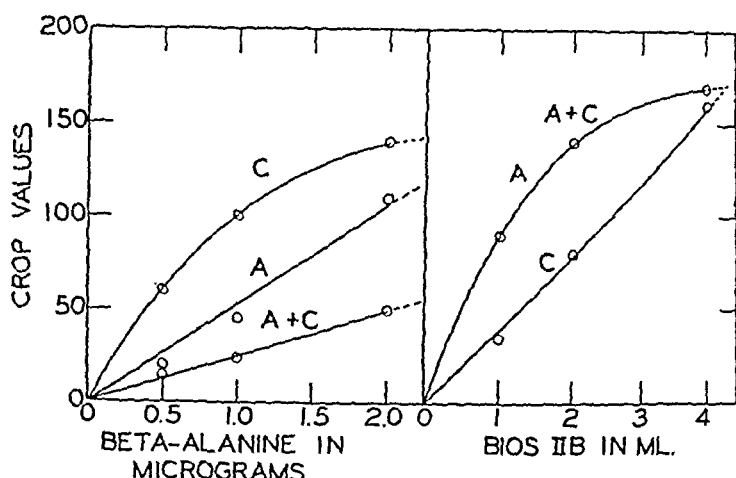
FIG. 1. Growth of *Saccharomyces cerevisiae*, Hansen Type B (Strain 4226), on ammonia nitrogen (*A*); carbamide nitrogen (*C*); and a mixture of the two (*A + C*).   To the left, effect of β-alanine increments in the presence of excess Bios IIB.   To the right, effect of Bios IIB increments with β-alanine in excess.

the mixture causes no interference and the crops are equal to the ammonia crops.

DISCUSSION

The assembled data indicate that both bios fractions, Bios IIA and Bios IIB, are essential for yeast growth.   This is true of all of the yeasts tested, and on both forms of nitrogen.   The mechanism for the assimilation of carbamide nitrogen is in some way more dependent on the amount of Bios IIB available; *i.e,* it requires more of it.   The presence of carbamide in the medium does not merely decrease the availability of Bios IIB, since growth on a mixture of carbamide and ammonium sulfate gives the higher crop in this case.

Similarly, the mechanism for the assimilation of ammonia nitrogen demands more β-alanine than for the assimilation of carbamide nitrogen.   The presence of ammonia nitrogen seems to affect the availability of the β-alanine, since ammonium sulfate decreases the carbamide crop in a mixture, as may be observed in Fig. 1.

SUMMARY

With each of eight cultures of *Saccharomyces cerevisiae* tested, four of Type A and four of Type B, the growth with ammonia and carbamide nitrogen is found to depend upon the bios supplement.

Considering the growth on ammonia nitrogen as a reference point, it is found that growth on carbamide nitrogen requires more Bios IIB but less β-alanine.

Growth on mixtures of the two nitrogen sources is described for one strain of *Saccharomyces cerevisiae* Type B. An incompatibility of the two is observed under certain conditions.

The effect of bios on nitrogen utilization is believed to indicate a physiological relationship.

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THERMODYNAMIC PROPERTIES OF SOLUTIONS OF AMINO ACIDS AND RELATED SUBSTANCES

VI. THE ACTIVITIES OF SOME PEPTIDES IN AQUEOUS SOLUTION AT TWENTY-FIVE DEGREES*

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Earlier studies in this series (10–13) included determinations of the osmotic and activity coefficients of solutions of several types of amino acids. In this report the investigation has been extended to include vapor pressure measurements of some peptides of glycine and alanine.

Method and Materials

The vapor pressures of the peptide solutions were measured by the isopiestic method described by Robinson and Sinclair (9) with sucrose as the reference standard. After suitable purification, the purity of the amino acids was checked by determination of the nitrogen content by a modified Kjeldahl method (6) or by repeated determinations of the solubility by a method employed in earlier studies (12).

Sucrose—The sucrose was a sample purchased from the Bureau of Standards.

Glycylglycine—This was purchased from Amino Acid Manufactures. The solubility in successive determinations was 1.764 and 1.766 moles per 1000 gm. of water.

dl-Alanylglycine—This was synthesized in this laboratory.[1] Nitrogen calculated, 19.2 per cent; found, 19.1.

[1] We are indebted to Dr. Florence R. White for the preparation and analyses of these samples.

Glycyl-dl-Alanine—This was synthesized in this laboratory.[1] Nitrogen calculated, 19.2 per cent; found, 19.1.

TABLE I

Concentrations, in Moles per 1000 Gm. of Water, of Isopiestic Solutions of Sucrose and Peptides at 25°

	m_S	m_A	m_S	m_A	m_S	m_A	m_S	m_A
Glycylglycine	0.2039	0.2166	0.3367	0.3701	0.565	0.657	0.966	1.199
	0.2085	0.2227	0.3491	0.3868	0.576	0.674	0.984	1.228
	0.2201	0.2340	0.3687	0.4085	0.590	0.687	1.033	1.300
	0.2336	0.2508	0.3764	0.4200	0.598	0.704	1.086	1.372
	0.2484	0.2687	0.4261	0.4813	0.637	0.751	1.100	1.398
	0.2631	0.2859	0.4460	0.503	0.643	0.758	1.143	1.453
	0.2792	0.3042	0.501	0.578	0.662	0.789	1.170	1.501
	0.2953	0.3235	0.508	0.588	0.753	0.706	1.199	1.530
	0.3139	0.3442	0.519	0.600	0.772	0.929	1.244	1.608
	0.3178	0.3488	0.525	0.607	0.881	1.075	1.264	1.626
			0.546	0.634	0.947	1.173	1.386	1.820
			0.562	0.614			1.427	1.868
dl-Alanylglycine	0.1860	0.1951	0.2831	0.3009	0.531	0.586	0.729	0.817
	0.1863	0.1960	0.3313	0.3563	0.550	0.609	0.750	0.841
	0.2031	0.2162	0.4866	0.536	0.553	0.612	0.813	0.913
	0.2085	0.2229	0.4940	0.544	0.598	0.661	0.870	0.984
	0.2173	0.2323	0.517	0.568	0.657	0.733	0.898	1.010
	0.2714	0.2887	0.522	0.573	0.697	0.782	0.992	1.130
Glycyl-*dl*-alanine	0.2011	0.2107	0.4075	0.4431	0.568	0.630	0.816	0.925
	0.2173	0.2293	0.4427	0.4829	0.594	0.661	0.838	0.950
	0.2783	0.2991	0.4624	0.508	0.598	0.661	0.869	0.994
	0.2901	0.3084	0.4968	0.548	0.615	0.687	0.882	1.002
	0.3127	0.3377	0.504	0.551	0.637	0.708	1.020	1.175
	0.3558	0.3824	0.517	0.568	0.698	0.785	1.043	1.205
	0.3569	0.3857	0.529	0.582	0.699	0.786	1.137	1.322
			0.540	0.598	0.752	0.850		
dl-Alanyl-*dl*-alanine	0.2085	0.2133	0.3127	0.3233	0.636	0.664		
	0.2250	0.2320	0.3558	0.3689	0.707	0.737		
	0.2285	0.2346	0.3666	0.3795	0.708	0.741		
	0.2563	0.2642	0.4863	0.503	0.783	0.818		
	0.2786	0.2874	0.561	0.585	0.796	0.836		
	0.2916	0.3004	0.562	0.584	1.007	1.054		
Triglycine	0.2103	0.2331	0.2306	0.2561	0.2547	0.2894	0.2792	0.3197
	0.2181	0.2423	0.2401	0.2694	0.2593	0.2924	0.2946	0.3378
	0.2267	0.2527	0.2482	0.2753	0.2760	0.3125		

dl-Alanyl-dl-Alanine—This was purchased from Amino Acid Manufactures. Nitrogen calculated, 17.3 per cent; found, 17.4.

Triglycine—We are indebted to Professor E. J. Cohn and Professor T. L. McMeekin of the Harvard Medical School for the sample of triglycine which was used without further purification.

It was assumed that the values of the osmotic coefficients of the sucrose used as a reference standard could be represented by the equation $\varphi = 1 + 0.084m + 0.0104m^2 - 0.00237m^3 + 0.000115m^4$. The data on which this is based are mentioned in a previous paper (11).
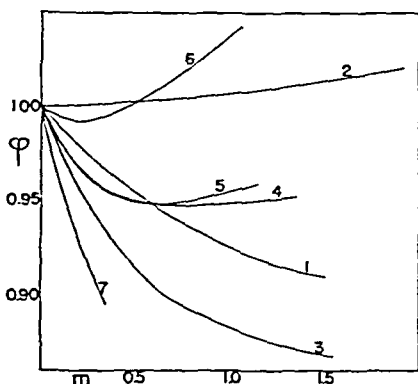
FIG. 1. Curve 1, glycine, Curve 2, *dl-α-alanine*, Curve 3, glycylglycine, Curve 4, glycyl-*dl*-alanine, Curve 5, *dl*-alanylglycine, Curve 6, *dl*-alanyl-*dl*-alanine, Curve 7, triglycine.

Results

The isopiestic molalities of the sucrose and the peptides are given in Table I. Smoothed values of m_S/m_A were used in the calculation of the osmotic coefficients from the relation $\varphi_A = \varphi_S(m_S/m_A)$. The results are shown in Fig. 1 and are represented, usually within ± 0.001, by the following equations.

$Glycylglycine$—$\varphi = 1 - 0.2628m + 0.2359m^2 - 0.1142m^3 + 0.0231m^4$
$Alanylglycine$—$\varphi = 1 - 0.2321m + 0.3398m^2 - 0.1517m^3$
$Glycylalanine$—$\varphi = 1 - 0.2047m + 0.2569m^2 - 0.1009m^3$
$Alanylalanine$—$\varphi = 1 - 0.0765m + 0.2038m^2 - 0.0883m^3$
$Triglycine$—$\varphi = 1 - 0.572m + 1.555m^2 - 2.35m^3$

In Fig. 1 the curves for the osmotic coefficients of glycine and of alanine are included for comparison.

The activity coefficients, γ, were calculated by the relations described in a previous paper (11)

$$2.3026 \log \gamma = 2\,am + 3/2\,bm^2 + 4/3\,cm^3 + 5/4\,dm^4$$

when $\varphi = 1 + am + bm^2 + cm^3 + dm^4$, and a, b, c, and d can be positive, negative, or zero. These data are summarized in Table II. The activities of the glycine peptides become progressively lower with the increase in length: the osmotic coefficient curve for glycylglycine has a much steeper slope than does that for glycine, while the triglycine values are still lower (see Fig. 1). Both the alanylglycine and the glycylalanine curves have a sharper

TABLE II

Activity Coefficients

m	Glycylglycine	Glycylalanine	Alanylglycine	Alanylalanine	Triglycine
0.1					0.910
0.2	0.912	0.935	0.929	0.981	0.852
0.3	0.878	0.912	0.906	0.979	0.804
0.5	0.827	0.882	0.878	0.985	
0.7	0.791	0.868	0.865	1.002	
1.0	0.744	0.854	0.855	1.036	
1.2	0.723	0.845			
1.5	0.696				
1.7	0.685				

initial slope than does glycine but soon level off and even turn upward beyond 0.6 M. In the case of alanine, lengthening the carbon chain with the interpolation of a peptide linkage to form alanylalanine also causes a drop in the more dilute part of the osmotic coefficient curve below that for the amino acid, but this is followed by a rise which becomes increasingly steep as the peptide solutions approach saturation.

DISCUSSION

From the results described above it appears that the osmotic coefficients, especially in dilute solutions, decrease with increased dipolar distance. This was also true for increasing dipolar distances in the amino acid series (11).

Cohn, McMeekin, Ferry, and Blanchard (2) have shown that,

for the α-amino acids, the molal change in the activity coefficient, $-(\log \gamma)/C$, is linearly related to the change in dielectric constant of the solution, D_0/D. A similar relation has been shown to hold, up to moderate concentrations, for other types of amino acids (11, 13). In Fig. 2 are shown the results of a similar calculation for the peptides. The values for the α-amino acids concerned are plotted for comparison. From the values of $-(\log \gamma)/C$ at $D_0/D = 1$, the relationships shown in Table III appear. When a single amino acid makes up a peptide, e.g. the series glycine, diglycine, triglycine, or alanine, alanylalanine, this function increases greatly with each added amino acid residue. The mixed dipeptides have values much larger than either component acid

TABLE III

Functions Derived from Change of Activity Coefficient with Concentration and Solvent

Compound	$\dfrac{-\mathrm{Log}\,\gamma}{C}$ at $\dfrac{D_0}{D} = 1$	$-\,\mathrm{Log}\,\dfrac{N_A}{N_0}$	$K_R{}^*$	$K_s{}^*$
Glycine............. ..	0.096	3.391	0.092	−0.004
Glycylglycine..........	0.241	4.367	0.227	−0.014
Triglycine.............	0.470	7.206	0.487	0.017
Alanine................	−0.005	2.856	0.010	0.015
Alanylalanine.......	0.073		0.203	0.130
Alanylglycine...........	0.208		0.293	0.085
Glycylalanine.......... .	0.189		0.243	0.054

but are between those for diglycine and dialanine; hence, the component amino acids, as well as their number and arrangement, influence this function. Cohn, McMeekin, Greenstein, and Weare (3) and McMeekin, Cohn, and Weare (7, 8) have investigated the activities of a number of amino acids and peptides by a comparison of their solubilities in water with those in certain organic solvents. Some of these results, expressed as the negative logarithm of the ratio of the solubility, in mole fractions, in alcohol to that in water, $-\log(N_A/N_0)$, are given in Table III. For the glycine series $\log(N_A/N_0)$ varies in the same way as does $(\log \gamma)/C$ with the number of peptide linkages contained. Solubility data for the other peptides are not available.

The values for the interaction constants (2) have been estimated for the peptides from the equation, $-(\log \gamma)/C = K_R{}^*(D_0/D) -$

$K_s{}^*$, where the interaction constant, $K_R{}^*$, is the slope of the line (see Fig. 2) and $K_s{}^*$, the salting-out constant, is the difference between $K_R{}^*$ and the intercept at $D_0/D = 1$. The values of the constants thus obtained are given in Table III. The interaction constants increase with the number of peptide bonds contained, but the salting-out constants show a less regular relation. Dialanine has a much larger $K_s{}^*$ than alanine, and triglycine
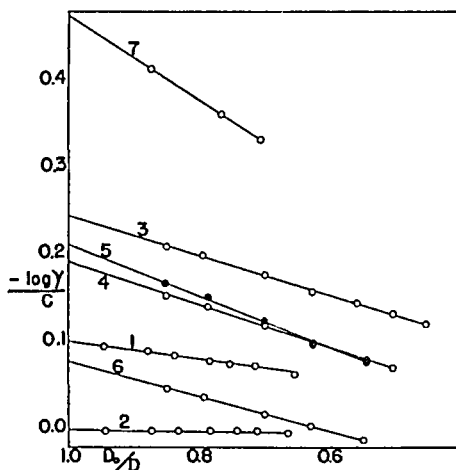
FIG. 2. Curve 1, glycine, Curve 2, *dl*-α-alanine, Curve 3, glycylglycine, Curve 4, glycyl-*dl*-alanine, Curve 5, *dl*-alanylglycine, Curve 6, *dl*-alanyl-*dl*-alanine, Curve 7, triglycine. The values for D_0 and those for D for Curves 1, 2, 3, and 7 are from Wyman (14) and from Wyman and McMeekin (15), those for Curves 4 and 5 from Greenstein and Wyman (5), while those for Curve 6 were assumed to be the same as those for Curve 5. The partial molal volumes used in calculating C from m for Curves 1, 2, and 3 are from Daniel and Cohn (4), for Curves 4 and 5 from Greenstein and Wyman (5), for Curve 7 from McMeekin, Cohn, and Weare (8), while that for Curve 6 was estimated by the method of Cohn *et al.* (1) to be 112.4.

than glycine or diglycine, but the values for the last two substances mentioned reverse the trend, although both are small negative quantities. A progressive increase in the interaction constant, $K_R{}^*$, with increasing dipolar distance was also found for the amino acids (11) but was of far less magnitude than in the case of the peptides; this suggests that long polypeptides and proteins have extremely high values for the interaction constant; that is, a very steep slope for the curve obtained by plotting $-(\log \gamma)/C$

against D_0/D. This is in accord with the large interaction constant of 1.90 found by Cohn, McMeekin, Ferry, and Blanchard (2) from a plot of the values of Richards for the solubility of hemoglobin.

SUMMARY

Isopiestic vapor pressure measurements have been made at 25° of aqueous solutions of glycylglycine, triglycine, alanylglycine, glycylalanine, and alanylalanine.

The osmotic and activity coefficients of these peptides have been calculated.

Certain relations between the activity coefficients and the dielectric constants of their solutions have been discussed.

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LIPID METABOLISM IN BRAIN AND OTHER TISSUES OF THE RAT*

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The primary object of the investigation described in this and
the following communications was the study of brain lipid metab-
olism with deuterium as an indicator. Nervous tissue is unique
in containing a larger amount of lipids than any other tissue;
fatty substances comprise over half of the non-aqueous constit-
uents of the whole brain, with the largest concentration in the
myelinated structures. Little is known concerning the metab-
olism or function of these major constituents of nervous tissue.
Recently developed labeling techniques offer the possibility of
yielding new and valuable information concerning the brain lipids,
and two indicators—radioactive phosphorus (1) and elaidic acid
(2)—have been employed in such investigations. The use of
P^{32} as an indicator in studies of lipid metabolism is restricted to
the phosphoric group, while with deuterium the metabolism of
all lipid constituents containing stable, carbon-bound hydrogen
may be studied.

In the present investigation two procedures were used: the
deuterium content of the unsaponifiable lipids and the fatty acids
of the brain was determined in adult, male rats at varying in-
tervals after the administration first of labeled fat, and secondly
of heavy water. Determinations were carried out by the same

procedures on other tissues (liver, intestine, and remaining carcass) for comparison with the brain findings.

The results obtained with the first of these procedures are described in this paper. Single, small, equal doses of linseed oil which had been partially hydrogenated with deuterium were administered to rats which were killed at intervals up to 48 hours thereafter. Whereas labeled fatty acids were present in liver, intestine, and even in the depot (carcass) fat in considerable

TABLE I

Distribution of Labeled Fatty Acids in Tissues after Administration* of Fat Containing Deuterium

Rat No.	Time after admin- istra- tion	Deuterium in fatty acids of				A† Administered fatty acids in fatty acids of				B‡ Portion of adminis- tered fatty acids in depots
		Brain	Liver	Intes- tine	Carcass	Brain	Liver	Intes- tine	Carcass	
	hrs.	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
6	8	0.02	1.21	0.92	0.05	0.3	18.8	14.3	0.8	32
7	8		0.51	0.41	0.03		7.9	6.4	0.5	25
8	16	0.02	0.93	0.24	0.06	0.3	14.4	3.7	0.9	15
9	16		0.67	0.15	0.06		10.4	2.3	0.9	37
3	24	0.03	1.06	0.29	0.14	0.5	16.4	4.5	2.2	30
4	48	0.02	0.54	0.10	0.06	0.3	8.4	1.6	0.9	22

* 1.5 cc. (1.33 gm. of fatty acids) of labeled fat were administered. The fatty acids contained 6.45 atom per cent deuterium.

$$\dagger\ A = \frac{\text{atom per cent } D_2 \text{ in fatty acids} \times 100}{\text{atom per cent } D_2 \text{ in administered fatty acids}}.$$

$$\ddagger\ B = \frac{A \times \text{depot fatty acids}}{\text{administered fatty acids}}.$$

amounts, only traces were found in the brain (Table I). This result indicates that the rate of fatty acid turnover is much slower in brain than in other tissues. McConnell and Sinclair (2) interpreted their somewhat analogous finding of a relatively small uptake of elaidic acid in brain phospholipids of young rats as indicating "that the selection of fatty acids ... in the building up of the constituent phospholipids of the tissues is much more rigorous in the brain than in the liver and muscles." A similar interpretation, if applied to our findings on the assumption that

the labeled acids were not utilizable by brain, would lack plausibility because many of the administered labeled fatty acids probably had the same structure as those native to the brain. The possibility remains that the brain synthesizes the fatty acids it needs for replacement and does not depend on an external source of supply. If so, the rate of turnover may not be as slow as the low deuterium concentrations appear to indicate (*cf.* the following paper (3)).

The findings are in general agreement with those obtained with essentially the same technique (except for a different lipid fractionation) and reported by Cavanagh and Raper (4) while this investigation was in progress. However, they observed significant amounts of deuterium 10 hours after administration (0.05 atom per cent in the acetone-soluble and 0.08 atom per cent in the acetone-insoluble lipids). The values had dropped to 0.025 and 0.03 atom per cent respectively at 24 hours.

Our results are in accord with those of Cavanagh and Raper in showing the highest concentrations of deuterium in the liver lipids. However, no clear cut relation to the time after administration of fat is evident from our data. Both maximum and minimum values were encountered in rats killed 8 hours after administration.

The findings on the intestine are presented as minimum values because, despite an attempt to remove all adherent fat, the percentages of fatty acids found (6 to 10 per cent moist weight) lead one to suspect that the removal was not complete. Since the concentration of deuterium in the intestine fatty acids was in all cases considerably higher than that found in the carcass, it is evident that it would have been still higher if the adherent depot fat had been completely removed.

Our data on the depot (carcass) fatty acids are not directly comparable with those of Schoenheimer and Rittenberg (5) and of Barrett, Best, and Ridout (6), because we used rats instead of mice, and because we measured the deuterium concentration in tissue lipids at relatively short intervals (maximum 48 hours) after the administration of a single small dose of labeled fat, while they fed it in the diet continuously, usually at a level of 15 or 20 per cent, over periods which in most instances were considerably longer. Both groups of investigators found that a

considerable proportion of the depot fat, up to almost one-half, was replaced by labeled dietary fat under these conditions. In the present experiments the percentage of labeled fatty acids in those of the depot fat must have been small (maximum found, 2.2 per cent), because the quantity of labeled fatty acids administered was small in comparison with that of the stores. However, the labeled fatty acids found in the depots, although comprising only a small part of the total in the stores, represented a large proportion of the quantity given (last column, Table I). This finding confirms for the rat the conclusion reached by Schoenheimer and Rittenberg (5) on the basis of an experiment in which mice were fed labeled fat at a 1 per cent level for 4 days; namely,

<div align="center">

TABLE II

Depot Fatty Acids

</div>

Rat No.	Weight of rats	Amount of fatty acids in carcass	Carcass fatty acids in animals
	gm.	*gm.*	*per cent*
6	307	53.7	17.5
7	367	71.5	19.5
8	337	21.7	6.4
9	320	53.4	16.7
3	200	18.2	9.1
4	245	31.4	12.8

that a large part of the diet fat, even when it is present in small quantities, may be deposited in fat tissues before it is burned. Our results indicate that the fat is deposited very soon after absorption; over one-fourth of the amount given was in the stores 8 hours after administration. Most of the difference between the amount given and that found in the depots must have been due to the fat burned. The amounts were too small, however, to increase the deuterium concentration of the body water, which was determined in all experiments, beyond the range of error of the analysis.

There was a large variation from rat to rat in the proportion of the administered fatty acids stored with no apparent relation to the time after administration; both maximum and minimum values occurred in rats killed 16 hours after administration. Since

the percentage of labeled fatty acids in those of the depots was the same in each of these rats, it is evident that the large difference in the proportion of administered fatty acids stored was related to an equally wide difference in the total amount of fatty acids in the depots of Rats 8 and 9 (Table II). The rat with the smaller stores deposited the smaller absolute amount of administered fatty acids.[1]

It is well known that rats, even when raised under identical conditions from the same stock, vary markedly in the amount of depot fat. We found this to occur (Table II) with our rats, taken from a colony of original Wistar stock which has been inbred for many years. Even larger variations among individual mice were found by Schoenheimer and Rittenberg (5), and Best *et al.* (6) reported large differences among the amounts of depot fat in mice kept under the same conditions even though the values were averages of seven to ten mice. Variability in the storage of fat apparently is a constitutional phenomenon, possibly related to a variable ability of the animal to burn or store fat, and must be considered in interpreting findings in investigations of fat metabolism.

EXPERIMENTAL

Preparation of Labeled Fat—The fat was prepared by hydrogenating linseed oil with deuterium in the apparatus of Rittenberg and Schoenheimer (7). It was a homogeneous liquid at body temperature. The fatty acids isolated from this fat contained 6.45 atom per cent deuterium.

Administration of Fat and Care of Rats—Adult male rats were selected from stock and 1.5 cc. of the tagged fat (1.33 gm. of fatty acids) were administered by stomach tube. Rats 3 and 4 were kept on a diet of dried bread for 8 days before fat administration and during the experimental period. No attempt was made to maintain these rats under constant environmental conditions. The remaining rats were given a low fat diet (No. 49) (casein (fat-

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[1] Cavanagh and Raper (4) gave a single dose of labeled fat to rats, as in the present experiments. However, it is impossible to calculate the proportion of administered fat in the depots from their data, as only an aliquot was analyzed.

free) 20, corn-starch 72.5, ryzamin-B[2] 1, spintrate 0.5, liver extract 1, and salt mixture[3] 5) and maintained for several days before and during the experiment in an incubator at a temperature of 25–26°. The incubator was equipped with a built-in blower and heating unit (controlled by a thermostat) at the top, but regulation at the desired temperature could be accomplished only by cooling by means of a large fan directed against water in a shallow pan placed on the floor. We adopted the foregoing technique in order to minimize the possible effect of environmental changes on fat metabolism.

Separation of Lipids—The rats were killed by decapitation and the tissues (brain (including the spinal cord to the thoracic region), liver, intestine,[4] and remaining carcass) were removed at once, weighed, and refluxed with 5 volumes of 10 N aqueous KOH for 18 to 20 hours. This procedure was adopted on the basis of an extended series of experiments designed to ascertain optimum conditions for saponification of brain lipids. The solution was transferred to a separatory funnel together with an equal volume of alcohol and extracted five times with petroleum ether. The combined extracts were washed twice with 2 N KOH and then with water (usually five times) until neutral. The KOH and the first two water washings were added to the water-alcohol solution which was acidified with 10 N H_2SO_4 and in most determinations (see below) extracted five times with petroleum ether. The extracts were washed with water until neutral (five to eight times).

The two petroleum ether solutions containing the unsaponifiable and fatty acid fractions were dried over Na_2SO_4, filtered, taken to dryness under CO_2, and brought to constant weight in a desiccator.

Considerable trouble was experienced in the extraction of fatty acids of the carcass because of the separation of large quantities of salt, and the following procedure was devised to overcome this difficulty. After acidification the salt together with the fatty

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[2] The ryzamin-B was supplied by Burroughs Wellcome and Company.

[3] Hawk, P. B., and Oser, B. L., *Science*, **74**, 369 (1931).

[4] Adherent fat was removed as completely as possible from the intestines, which were then opened and washed thoroughly with water. The excess water was removed with filter paper before weighing for analysis.

acids is filtered off on a Buchner funnel. The filtrate should be crystal-clear; if the first portion is cloudy it is returned to the funnel. The salt is sucked as dry as possible and the clear filtrate, which was proved to contain negligible quantities of fatty acids, is discarded. The salt is washed several times with acetone which is allowed to remain in contact with the salt for a few minutes each time before sucking off. To be certain of complete extraction the salt is finally washed into a clean flask with several portions of petroleum ether. Most of the acetone is removed on the steam bath under CO_2 and the remaining aqueous solution on which a layer of fatty acids is floating is transferred to a separatory funnel. The flask is washed with the petroleum ether used in washing the salt and the washings are added to the contents in the funnel. The aqueous solution is washed three times with petroleum ether, the combined extracts are washed with water until neutral, dried, filtered, and evaporated to dryness under CO_2.

It is a matter of general knowledge that a portion of unsaponifiable fractions kept in the dry state becomes insoluble in petroleum ether. Hence these fractions were redissolved in petroleum ether and thus kept until they could be analyzed for deuterium.

To our surprise some of the unsaponifiable fractions contained significantly large concentrations of deuterium, a result which, taken at its face value, would indicate a formation of unsaponifiable substances from fatty acids. However, deuterium was found in appreciable concentrations in the unsaponifiable fractions only in samples (mostly from the liver) in which the fatty acids contained a large concentration, and we suspected that despite the care taken to wash out fatty acids enough had remained to account for the deuterium found. Hence some unsaponifiable fractions were reextracted with alkali. A small amount of fatty acids was obtained and the deuterium concentration fell almost to zero. To test the point further labeled fat was administered to eight rats which were killed 41 hours later. The brains, livers, and intestines were pooled and worked up as described above. The unsaponifiable fractions were "washed out" with an excess of palmitic acid according to the procedure used by Schoenheimer and Rittenberg to remove saturated from unsaturated fatty acids (8). These fractions contained negligible concentrations of

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deuterium. Because of these findings, data obtained on unsaponifiable fractions are not included.

Determination of Deuterium—The deuterium content of the lipid fractions was determined by the falling drop procedure according to Keston, Rittenberg, and Schoenheimer (9). A modification of trap *e* ((9) Fig. 2) may be mentioned. Our traps are made with a conical tip, similar to that of the usual 15 cc. centrifuge tubes, instead of the graduated cylinder, which is usually not necessary. With this modification it is possible to recover small samples of water more completely.

All determinations of dropping time were repeated on at least 2 drops (usually 3 or more). All samples were then redistilled

TABLE III
Error of Deuterium Determination in Fat

Sample No.	Deuterium in Fat 1	Deuterium in Fat 2
	atom per cent	*atom per cent*
1	0.036	0.063
2	0.033	0.059
3	0.040	0.064
4	0.037	0.060
5	0.039	0.065
6	0.037	
Average......................	0.037	0.062
Standard deviation...........	±0.0025	±0.0026

and the dropping time was determined again. Excellent agreement among replicates and between the determinations before and after distillation was obtained. However, the greatest potential sources of error reside in the combustion of the sample and the purification of the water (10). To obtain an estimate of the over-all error[5] several samples of two fats containing deuterium

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[5] The potential error due to a variable proportion of isotopes in the oxygen used for combustion is not included. Dole (10) has shown that there is a difference of 6.0 parts per million between water made from oxygen of the air and water made from oxygen originally in water. This factor did not enter into our experiments, since liquid air oxygen was used throughout. Commercial oxygen obtained in fractional distillation of liquid air is increased in atomic weight by 2.2 parts per million (10, 11). A variation of this magnitude would not influence the interpretation of our data.

were analyzed. Fats with small concentrations, prepared by diluting the fat used in these studies with olive oil, were selected because it is in the interpretation of such results that the greatest difficulty is encountered; *e.g.*, the findings in carcass fatty acids (Table I). It is evident from the results (Table III) that the procedure under our conditions gives reproducible results within a narrow range of variation.

SUMMARY

The distribution of labeled fatty acids in the brain, liver, intestine, and remaining carcass of adult rats was determined at varying intervals (from 8 to 48 hours) after the administration of a single small amount of deuterium-containing fat.

Only traces of the labeled fatty acids were found in the brain.

The largest concentrations were found in the liver and intestine.

Although the concentration was small, a large but variable proportion of the total amount given was found in the depot (carcass) fatty acids. The variation bore no evident relation to the time after administration.

Attention is called to constitutional variation in the amount of fat in the stores as a factor to be considered in interpreting studies of fat metabolism.

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A STUDY OF THE SYNTHESIS AND DEPOSITION OF LIPIDS IN BRAIN AND OTHER TISSUES WITH DEUTERIUM AS AN INDICATOR*

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New York)

In the preceding investigation (1) only traces of deuterium-labeled fatty acids were found in the brain up to 48 hours after administration to adult rats. In the investigation here described the deuterium concentration of rat body fluids was raised by the injection of deuterium oxide and maintained for varying periods of time at the increased concentration by adding heavy water to the drinking water. The deuterium concentration in the unsaponifiable and fatty acid fractions of brain, liver, intestine, and the remaining carcass was determined at the end of the experimental periods.

If under these experimental conditions little or no deuterium is found in a lipid fraction from an organ, while at the same time large concentrations are present in the same fraction from other tissues of the body, it is reasonable to conclude that the rate of synthesis or deposition of the lipids comprising that fraction is very slow in that organ. This was true of the brain unsaponifiable fraction in our experiments (Table I). On the other hand, the deuterium level in the brain fatty acid fractions (Table II) was comparable with that found in the depot (carcass) fatty acids. This result, which shows that the rate of turnover or replacement of fatty acids in the adult rat brain, though slow, is

by no means negligible, is not in conflict with the finding of a very small uptake of labeled fatty acids in the preceding study (1).

TABLE I

Deuterium Content of Unsaponifiable Lipids of Tissues of Adult Male Rats Given Heavy Water

Rat No.	Duration of experiment	D₂ in body fluids	Deuterium in unsaponifiable lipids of				Hydrogen from body fluids in unsaponifiable lipids of			
			Brain	Liver	Intestine	Carcass	Brain	Liver	Intestine	Carcass
	days	atom per cent	atom per cent	atom per cent	atom per cent	atom per cent	per cent	per cent	per cent	per cent
5	4	1.22	0.00	0.20*	0.05*	0.23	0.0	16.4	4.1	18.9
10	4	1.05	0.03	0.15*	0.13*	0.19	2.8	14.0	12.1	18.1
11	4	1.10				0.22				20.0
12	6	1.20	0.03	0.17*	0.20*	0.32	2.6	14.5	17.0	26.7
13	6	1.15				0.30				26.1
1	7	1.36	0.02* (?)	0.25*	0.23*	0.20	1.5 (?)	18.4	16.9	14.7

* Cholesterol was added to these samples before combustion to provide sufficient water for analysis.

TABLE II

Deuterium Content of Fatty Acids of Tissues of Adult Male Rats Given Heavy Water

Rat No.	Duration of experiment	D₂ in body fluids	Deuterium in fatty acids of				Hydrogen from body fluids in fatty acids of			
			Brain	Liver	Intestine	Carcass	Brain	Liver	Intestine	Carcass
	days	atom per cent	atom per cent	atom per cent	atom per cent	atom per cent	per cent	per cent	per cent	per cent
5	4	1.22	0.06	0.15	0.03	0.02	4.9	12.3	2.5	1.6
10	4	1.05	0.06	0.34	0.15	0.09	5.6	32.4	14.3	8.6
11	4	1.10		0.35	0.13	0.09		31.8	11.8	8.2
12	6	1.20	0.08	0.29	0.13	0.07	6.8	24.2	10.8	5.8
13	6	1.15		0.43	0.23	0.15		37.4	20.0	13.0
1	7	1.36	0.09	0.26	0.22*	0.16	6.6	19.1	16.2	11.8

* Palmitic acid was added to this sample before combustion to provide sufficient water for analysis.

A direct comparison cannot be made, as the experimental conditions were different, but the results of the two experiments are in accord with the hypothesis that the brain synthesizes the fatty

acids it needs, wholly or in large part, and does not depend on an external source of supply.[1]

The question arises, what quantity of newly synthesized fatty acids do the deuterium values represent? If it be assumed that the same proportion (*i.e.* about one-third[2]) of body water hydrogen enters the molecules of the synthesized brain fatty acids of rats as apparently enters the fatty acids of mice, then about 20 per cent of the total brain fatty acids had been replaced within a week. This calculation is subject to criticism, first because there was a considerable difference between saturated and unsaturated fatty acids in uptake of deuterium in Rittenberg and Schoenheimer's experiments,[2] the value of one-third being an average, and secondly because the transfer of findings on the total fatty acids of mice to those of the rat brain may not be justified. Despite these uncertainties[3] it is probable that the estimated rate of turnover is of the right order of magnitude.

The liver fatty acids had a considerably higher deuterium level than any other tissue examined; in four of the six experiments approximately one-third of the hydrogen atoms originated in the body fluids. This result would indicate[2] that most of the liver

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[1] To test this hypothesis we plan to administer individual, labeled fatty acids, characteristic of the brain, to rats.

[2] The proportion of hydrogen atoms from the body water entering the molecules of synthesized fatty acids can be estimated in experiments in which the body fluids are enriched in deuterium and which are continued to the point where the maximum deuterium content is approached, and where it may, therefore, be assumed that most of the fatty acids present were synthesized during the experiment. In such an experiment Rittenberg and Schoenheimer (2) found that about one-third of the hydrogen atoms of the total fatty acids of mice had come from the body water.

[3] The concentration of deuterium in the body fluids on which the calculations must be based may fluctuate during the experiments. We followed the procedure used by Schoenheimer and Rittenberg with mice in injecting an amount of heavy water calculated to bring the body water to a concentration of 1.5 atom per cent deuterium at the beginning and in giving the rats drinking water containing 2.5 atom per cent of deuterium. It will be seen from Tables I and II that the concentrations found in the body fluids at the end of the experiments were usually considerably below the expected 1.5 atom per cent. If the concentration was actually brought to the calculated 1.5 atom per cent at the start, there must have been a gradually decreasing concentration of deuterium in the fluids in which the synthesis was taking place, and calculations based on the final concentration will be somewhat in error.

fatty acids had been synthesized during the experiment. From Sinclair's finding (3) that the turnover of the phospholipid fatty acids of the liver is virtually complete in 1 day and the experiments of Bernhard and Schoenheimer (4), indicating "that the half life of the average saturated fatty acid molecule in the liver [of mice] is very short, probably about 1 day," it would be expected that practically all of the fatty acids would be replaced within the shortest period (4 days) studied in this investigation. If so, the concentration of deuterium in the liver fatty acids must represent the degree of replacement by newly synthesized fatty acids and by those coming from the stores in which the concentration of deuterium was low. Thus in the two rats (Nos. 1 and 5) in which the deuterium level was relatively small a considerable part of the liver fatty acids had apparently come from the stores, even though the rats were receiving adequate amounts of a high carbohydrate diet (cf. (5)).

In contrast with the fatty acids the deuterium level of the unsaponifiable fraction of the liver was lower on the average than that from the carcass and about the same as that from the intestine (except in Rat 5). Most surprising was the large concentration of deuterium in the carcass unsaponifiable fractions. A detailed study of these fractions (6) revealed that the deuterium concentration of the non-cholesterol alcoholic portion was almost twice as great as that of the other constituents.

The finding of relatively large amounts of deuterium in the intestine fatty acids and unsaponifiable lipids[4] indicates that the intestine plays an active rôle in lipid metabolism. It must be remembered that these rats received a diet containing almost no fat. The copious secretion of lipids into the gut found by Sperry and Angevine (7) in dogs is probably related to activity of the intestine in endogenous lipid metabolism shown by the present experiments.

The results with the carcass fatty acids are in fairly close agreement with those reported by Rittenberg and Schoenheimer (2)

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[4] Despite efforts to remove it completely some adherent depot fat was probably included (see the preceding paper). The amount of intestine fatty acids was especially high (9.2 per cent) in Rat 5 in which the lowest deuterium concentration was found. The values reported may be regarded, therefore, as minimum.

in mice. However, the concentration of deuterium, corrected to the same body water percentage, tends to be a little lower (as is to be expected from the lower metabolism of the rat). There was considerable variation in both series.

Enough water containing 50 per cent D_2O to bring the body fluids to a calculated concentration of 1.5 atom per cent was injected into adult, male rats. From that time water containing 2.5 atom per cent of deuterium was given the rats to drink. The analytical procedure was the same as that described in the preceding paper (1). In some instances the lipid fractions were too small to provide sufficient water for redistillation after the first series of determinations. Such unsaponifiable fractions were diluted with one-half to one part by weight of cholesterol before combustion (Table I); palmitic acid was added in the same way to fatty acid fractions when necessary (Table II).

SUMMARY

The concentration of deuterium in the unsaponifiable and fatty acid fractions of the brain, liver, intestine, and carcass of adult, male rats was determined after periods of 4 to 7 days during which the body fluids were enriched with deuterium through the administration of heavy water.

In the brain unsaponifiable fraction the concentration of deuterium was little above the error of the method, while all other tissues contained large concentrations. It is concluded, therefore, that the lipids comprising this fraction are replaced at a very slow rate.

The concentration of deuterium in the fatty acid fraction of the brain on the other hand was comparable with that found in the depot (carcass) fatty acids, and it was estimated that as much as one-fifth of the brain fatty acids may be replaced in a week.

In the liver fatty acids the concentration of deuterium was higher than in any other tissue.

The concentration of deuterium in the liver unsaponifiable fraction was less on the average than that of the carcass and no greater than that of the intestine. Thus the liver does not appear to have

the same dominant rôle in the metabolism of unsaponifiable substances as it does in fatty acid metabolism.

Relatively large concentrations of deuterium in both the unsaponifiable and fatty acid fractions of the intestine direct attention to this organ as an active participant in endogenous lipid metabolism.

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LIPID METABOLISM IN BRAIN DURING MYELINATION*

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In the preceding investigations (1, 2) the rate of turnover of
lipids was found to be slower in the brain of adult rats than in
other organs (liver and intestine) studied for comparison. In this
investigation lipid metabolism was studied during the time of its
greatest activity in nervous tissue—the period known to mor-
phologists as that of myelination.

The process of myelination has been little studied from the
biochemical point of view since the classical investigation of Koch
and Koch (3) on rat brain. Indeed the only studies of brain
lipids during the period when myelination takes place, aside from
that of Koch and Koch, appear to be a few analyses of formalin-
fixed tissue by Smith and Mair in puppies (4) and in man (5),
determinations in three infants by MacArthur and Doisy (6),
and some analyses of phospholipid by McConnell and Sinclair
(7). Koch and Koch found a rapid increase, both relative and
absolute, of phosphatide between the 10th and 20th day of extra-
uterine life. From the 20th day on the percentage remained
constant, though there was a gradual increase in the absolute
amount with growth of the brain. Cerebrosides doubled in
percentage amount between the 20th and 40th day. The per-
centage of cholesterol fluctuated widely and the data are of little
value as the determinations were made by difference. The find-
ings were correlated with the process of myelination which from

histological studies (*cf.* Watson (8)) starts around the 13th or 14th day in the major structures of the rat brain.

In the present study heavy water was injected into mother rats and their young 15, 26, and 36 days after birth, and the drinking water was enriched as in the preceding investigation. The heavy water régime was continued for 4 days, after which both the young and the mother rats were worked up as described in the preceding investigation, except that the spinal cord was not included with the brain because, according to Watson (8), myelination starts much earlier in the cord than in the brain. The nursing young of the first group obtained deuterium in the milk. This procedure was employed by Ussing (9) in studying metabolism with deuterium in nursing rats. ·

In undertaking the investigation we were particularly interested in ascertaining the source of the lipids deposited in the brain during myelination. The findings in the rats which received heavy water from the 15th to the 19th day of life (Tables I and II) indicate that the lipids deposited during this period of most active myelination are synthesized in the brain. It may be assumed from the data of Koch and Koch and from unpublished experiments that the absolute amount of lipids in the brain approximately doubles during this period and that about half of the lipids analyzed was present at the beginning of the experiment. If it be assumed that the turnover in this portion proceeded at the same slow rate found in adult male rats in the preceding investigation (2), and in the mother rats of the present study, the large concentrations of deuterium found in the unsaponifiable and fatty acid fractions of the brain of the 19 day-old rats must represent in greatest part newly deposited lipids. Since only about half of the lipids had been deposited during the experiment, their deuterium content must have been approximately twice as great as the concentrations measured and considerably higher than was found in any other tissue of the mothers or of the young rats. The deposited lipids could have originated, therefore, only in synthesis in the brain itself. It will be noted that, if the foregoing calculation is valid, the uptake of hydrogen from the body fluids in the fatty acids deposited in the brain in the 19 day-old rats must have exceeded considerably the value of one-third (see foot-note 2 (2)). The same result was obtained in the liver fatty acids of the 30 and 40 day-old rats (see below).

Koch and Koch (3) showed that different lipids were deposited in the brain over different periods and at different rates during

TABLE I

Deuterium Content of Unsaponifiable Lipids of Tissues of Young Rats and Their Mothers Given Heavy Water for 4 Days

Rats	No. of rats	Age when killed	D$_2$ in body fluids	Deuterium in unsaponifiable lipids of				Hydrogen from body fluids in unsaponifiable lipids of			
				Brain	Liver	Intestine	Carcass	Brain	Liver	Intestine	Carcass
		days	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Mothers....	2		1.15	0.04*	0.25	0.24*	0.25	3.5	21.7	20.9	21.7
Young.....	14	19	0.95	0.20	0.13*	0.22*	0.29	21.1	13.7	23.2	30.5
Mother.....	1		1.22	0.05*	0.15	0.22*	0.27	4.1	12.3	18.0	22.1
Young.....	7	30	1.26	0.09	0.23	0.30	0.36	7.1	18.3	23.8	28.6
Mother.....	1		1.14	0.04*	0.26*		0.24	3.5	22.8		21.1
Young.....	6	40	1.07	0.06*	0.19*	0.26*	0.35	5.6	17.8	24.3	32.7

* Cholesterol was added to these samples before combustion to provide sufficient water for analysis.

TABLE II

Deuterium Content of Fatty Acids of Tissues of Young Rats and Their Mothers Given Heavy Water for 4 Days

Rats	No. of rats	Age when killed	D$_2$ in body fluids	Deuterium in fatty acids of				Hydrogen from body fluids in fatty acids of			
				Brain	Liver	Intestine	Carcass	Brain	Liver	Intestine	Carcass
		days	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Mothers....	2		1.15	0.06	0.34	0.19	0.07	5.2	29.6	16.5	6.1
Young.....	14	19	0.95	0.24	0.32	0.34	0.16	25.3	33.7	35.8	16.8
Mother.....	1		1.22	0.08*	0.35	0.16*	0.22	6.6	28.7	13.1	18.0
Young.....	7	30	1.26	0.16	0.50	0.45*	0.21	12.7	39.7	35.7	16.7
Mother.....	1		1.14	0.08	0.38	0.25*	0.08	7.0	33.3	21.9	7.0
Young.....	6	40	1.07	0.11	0.55	0.35	0.21	10.3	51.4	32.7	19.6

* Palmitic acid was added to these samples before combustion to provide sufficient water for analysis.

myelination. It will be seen from Tables I and II that the concentration of deuterium in the brain lipids, though high in comparison with that of adult rats (2), was considerably less in the

30 day-old than in the 19 day-old rats, and still less in those 40 days old. If it be assumed that a turnover of lipids, independent of new deposition, occurs in these young rats at the same rate found in adults, and if correction be made for this factor, then the rate of deposition between the 36th and 40th day was very small. This conclusion is in agreement with histological evidence (8) which indicates that myelination approaches completion by the 40th day of life. Superficially, the rate of deposition of unsaponifiable material appears to fall off more rapidly than that of the fatty acids. However, the difference largely disappears when correction is made for turnover.

It is of interest to compare the findings in other tissues of the young rats with those obtained in adult animals (2). For the most part the relationships were the same, though in general the deuterium uptake was considerably higher in the young than in the adult rats. The greater rate of metabolism, thus indicated, would be expected in a young growing animal. Values for the fatty acids in the liver are particularly striking; half of the hydrogen apparently originated in body fluids between the 36th and 40th day of life. This value is probably somewhat too high because of variation in the deuterium content of body water (see foot-note 3 (2)); but even after a reasonable allowance for this error the value is still well above one-third—the factor indicated by the data of Rittenberg and Schoenheimer (10) as the proportion of hydrogen from the body water in the total fatty acids synthesized by the mouse (see foot-note 2 (2)). This proportion was an average of the uptake of hydrogen in the saturated fatty acids (about 43 per cent) and in the unsaturated fatty acids (about 28 per cent). The difference between the saturated and unsaturated fatty acids in Rittenberg and Schoenheimer's experiment was not fully explained, though attention was called to the possibility that the maximum uptake of hydrogen had not been reached in the unsaturated fatty acids. In a recent publication Bernhard and Schoenheimer (11) confirmed the greater uptake of hydrogen in the saturated than in the unsaturated fatty acids and pointed out that the difference may be due to the presence of "inert" fatty acids with two and three double bonds (not regenerated and therefore not containing deuterium) in the unsaturated fraction. The high uptake of hydrogen in the *total* fatty acids

of the liver and probably of the brain (see above) in our young rats may indicate either that the "inert" (essential) fatty acids are present in small amount or are synthesized at a rapid rate in early life. Sinclair (12) obtained evidence indicating that growing rats have a considerable ability to synthesize the essential fatty acids.

The high values in the intestine fatty acids emphasize the active rôle of this organ in lipid metabolism. The result in the 19 day-old rats may have been influenced to some extent by the absorption of labeled fatty acids from the mothers' milk but the 30 day-old rats were not observed to nurse during the experiment and the 40 day-old rats were separated from the mother.

The active metabolism of carcass unsaponifiable substances is brought out again; as in the adult rats the concentration of deuterium in this fraction was higher on the average than in any other tissues (cf. (13)).

A comparison of the findings in the mother rats with those obtained in the adult males (2) reveals good agreement on most points. The turnover of the brain unsaponifiable substances appears to have been a little higher in the mothers but, as in the male rats, it was much smaller than in any other tissue. A high value for the carcass fatty acids in the mother of the 30 day-old rats may also be noted. The mothers of the 19 day-old rats exhibited fatty livers grossly, with a fatty acid content of 16 per cent moist weight. It is probable that this was associated with lactation on a diet containing almost no fat and low in lipotropic factors. The deuterium content indicates that most of this fat had been synthesized during the 4 day experiment.

EXPERIMENTAL

Pregnant rats were placed on Diet 49 (1) about a week before parturition and continued on this low fat diet throughout the experiments. At the ages of 15, 26, and 36 days heavy water was injected into the young rats and into the mothers as in the preceding investigation and water containing 2.5 atom per cent of deuterium was given the rats to drink for the following 4 days. The 15 and 26 day-old rats were caged with their mothers; the 36 day-old rats were placed in a separate cage at the start of the experiment and given Diet 49. At the end of the experiments

the rats were worked up as described in the preceding papers (1, 2), the tissues from the young rats being pooled for analysis.

SUMMARY

The deposition of lipids in the rat brain during myelination was investigated with the aid of deuterium as an indicator.

Unsaponifiable lipids and fatty acids are deposited at a rapid rate from the 15th to 19th day of extrauterine life. The rate is considerably less from the 26th to the 30th day, and still lower from the 36th to the 40th day. If allowance is made for turnover comparable with that found in adult rats, no marked differences in the rates of deposit of the unsaponifiable and fatty acid fractions are evident.

The data indicate that lipids deposited between the 15th and 19th day of life are synthesized in the brain itself.

The relationships among rates of lipid metabolism in the liver, intestine, and remaining carcass were in general the same as were found in adult rats, but in most instances the deuterium uptake was considerably higher in the young than in the adult rats.

Determinations on mother rats agreed closely on most points with the findings in adult male rats (2).

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PHOSPHORUS CHANGES DURING THE ABSORPTION OF OIL AND GLUCOSE

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These experiments on the relationship of phosphorus to the metabolism of fat and glucose were part of a general plan to test the hypothesis proposed by Verzár (1) that the major symptoms of sprue, the faulty absorption of fats and carbohydrates, may be due to a breakdown in the phosphorylating mechanism.

With the exceptions of the changes in the blood serum and muscles after glucose ingestion the normal relationships of the phosphorus changes in the body to the ingestion of fat and glucose have been very little studied. The present work is a report of these normal changes.

The first phase of the work was a study of the changes in serum and urinary inorganic phosphate in humans after the ingestion of olive oil and glucose. The second phase was an investigation of the phosphate changes in the duodenal mucosa, liver, and kidney of swine during the absorption of cottonseed oil and glucose.

Procedure and Methods

The serum and urinary phosphate studies were conducted on humans. The subjects were either laboratory workers or patients at Duke Hospital who had recovered from minor ailments or accidents and were about to be discharged.

Except when stated otherwise, the subjects remained in bed during the entire course of the experiment and for at least 15 hours before. At 7.00 a.m., 13 hours after the last meal, urine

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was voided and the subject received a glass of water. Just before 8.00 a.m. urine was voided again and immediately afterward the test meal and a glass of water were given. Blood and urine samples were taken, and a glass of water was given at 8.30, 9.00, 10.00, 11.00 a.m., and in the experiments with oil at 12.00 noon. The test meal was either 50 gm. of glucose in a glass of water with the juice of half a lemon or 50 gm. of olive oil.

Three subjects were given an intravenous glucose tolerance test also. 25 gm. of glucose were injected as a 50 per cent solution and the serum phosphorus determined $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, and 3 hours afterwards. The urinary phosphorus was determined at the same times and intervals as it was for the test after oral administration of glucose.

The swine used in the studies were pure bred Duroc-Jerseys that had been on experiment to determine the relative values of different protein supplements in their nutrition. They had all been on the same diet for several weeks before these experiments. They were about 7 months old and weighed approximately 225 pounds. Three groups of five animals each were used. One group was slaughtered in the fasting state. One group was fed 200 gm. of glucose 1 hour and the other 200 gm. of cottonseed oil 2 hours before being slaughtered. The animals were held on their backs and the oil or glucose, the latter as a 50 per cent solution, poured into their mouths. They swallowed readily.

The swine were killed by exsanguination and the tissues frozen with solid carbon dioxide as soon as possible. 5 gm. samples of frozen tissues were weighed to the nearest decigram, ground in a mortar with 10 per cent trichloroacetic acid without sand, and transferred to 50 cc. volumetric flasks by means of dropping pipettes. They were allowed to stand a few hours with occasional shaking, filtered, and placed in the ice box overnight. The determinations were made the next day. 5 gm. were weighed for moisture determination.

All phosphorus analyses were made according to the Fiske and Subbarow procedure (2) except that the perchloric acid digestion described by King (3) was used for the total phosphorus determination. The difference between the total acid-soluble phosphorus and the inorganic phosphorus will be referred to as "ester" phosphorus.

DISCUSSION

In four experiments on three individuals the phosphorus excretion in the fasting state was shown to be at a minimum between 9 and 11 a.m. This is in agreement with the values given in the literature (4).

Table I shows the effect of the ingestion of 50 gm. of olive oil on the rate of phosphorus excretion in the urine. All nine experiments show a pronounced decrease in phosphorus excretion, the maximum decrease occurring 1 hour after the ingestion of the oil. This decrease is independent of the diurnal change, being much

TABLE I

Effect of Ingestion of 50 Gm. of Olive Oil upon Serum Inorganic Phosphorus and Rate of Phosphorus Excretion in Man

Hrs. after oil	Time	Serum phosphorus				Rate of excretion		
		Sample experiment	Extremes	Mean	Sample experiment	Extremes	Mean
	a.m.	*mg. per cent*	*per cent change*	*per cent change*	*mg. per hr.*	*per cent change*	*per cent change*
Fasting	7-8	4.00			22		
0.5	8.30	3.70	−7.5 to 0.0	−4.3	11	−84 to 0	−42
1.0	9.00	3.54	−11.5 " −4.1	−7.9	10	−90 " −55	−74
2.0	10.00	3.94	−7.9 " +0.9	−2.3	16	−71 " +75	−40
3.0	11.00	4.04	−4.7 " +5.7	+0.5	16	−73 " +19	−18
4.0	12.00	3.92	−4.7 " +5.6	+0.8	26	−43 " +118	+42

greater and occurring earlier. Youngburg (5) determined the urinary phosphorus excretion after normal breakfasts and after similar breakfasts to which olive oil had been added. On the basis of the total excretion for 7 hours after the meal he concluded that there was an increase rather than a decrease in the amount of phosphorus excreted. However, he recorded the excretion at hourly intervals and his data show that in three of his four experiments the phosphorus excreted in the 1st hour was reduced by more than twice as much after the fat meal as after the normal meal.

The effects of the ingestion of olive oil on the serum inorganic phosphate are also recorded in Table I. In seven of the eight

TABLE II

Effect of Glucose Administration upon Rate of Phosphorus Excretion in Man

Hrs. after sugar	Time	Ingestion of 50 gm. glucose							Injection of 25 gm. glucose		
		In bed			Walking				In bed		
		Sample experiment	Extremes	Mean	Sample experiments		Extremes	Mean	Sample experiment	Extremes	Mean
	a.m.	*mg. per hr.*	*per cent change*	*per cent change*	*mg. per hr.*	*mg. per hr.*	*per cent change*	*per cent change*	*mg. per hr.*	*per cent change*	*per cent change*
Fasting	7–8	27			29	30			11		
0.5	8.30	36	+100 to −55	+51	30	50	+67 to −6	+24	15	+67 to +17	+37
1.0	9.00	50	+305 " +17	+99	31	40	+33 " +6	+15	26	+137 " +4	+66
2.0	10.00	27	+121 " −61	+54	15	25	+15 " −48	−21	12	+13 " +1	+7
3.0	11.00	29	+47 " −75	−10	11	35	+17 " −62	−20	11	+35 " −22	+3

experiments performed there was a significant drop in this value $\frac{1}{2}$ hour and in all the experiments 1 hour after the ingestion of the oil. In a fasting, resting person the serum inorganic phosphorus remains constant in the morning (6, 7).

Table II shows the changes in urinary phosphorus excretion after oral and intravenous administration of glucose. The six subjects who remained in bed were awake but at complete rest during the entire course of the experiment. They had been in bed from 13 to 15 hours before the experiments began. Four experiments were performed on three persons who were standing and walking about the laboratory during the course of the experiments. The three subjects who received the intravenous injections of glucose remained in bed.

In all thirteen of the experiments there was an increase in the phosphorus excretion during the second half hour after the glucose administration. It was slight in a few cases but very marked in all the others. This increase has an added significance when it is recalled that in the fasting state there is a pronounced decrease in urinary phosphorus excretion at the same hour (4). This increase in phosphorus excretion after glucose administration is contrary to the commonly accepted belief. A study of the literature, however, shows that there is no unanimity of opinion on this subject. Katayama (8) and Wolbergs (9) report increased excretion of phosphate after glucose ingestion in man. Blatherwick, Bell, and Hill (10) in five experiments out of ten showed an increase in phosphorus excretion after glucose ingestion by human subjects. The data of Sokhey and Allen (11) which are often quoted as showing a decrease in urinary phosphate after administration of glucose by stomach tube to dogs actually show an increase in two out of three normal animals during the first 3 hour period. Bachmann, Haldi, and Ensor (12) could find no change in the urinary phosphorus excretion after the ingestion of glucose by humans.

On the other hand, Perlzweig, Latham, and Keefer (13), Barrenscheen and coworkers (14), and Wierzuchowski and coworkers (15) found a decrease in phosphorus excretion after glucose ingestion. Fiske (16) found a decrease in urinary phosphorus after sucrose ingestion.

Harrop and Benedict (17), Sokhey and Allen (11), Blatherwick and coworkers (10), Wigglesworth, Woodrow, Smith, and Winter

(18), Gortz (19), and many others have shown conclusively that the administration of insulin does lower phosphorus excretion in the urine.   It does not follow, however, that the administration of insulin and sugar should have the same effect upon urinary phosphorus.   Insulin produces a hypoglycemia and glucose a hyperglycemia.   Iversen and Jacobsen (20) have shown that with a rising glycemia there is an increased elimination of phosphorus and that a lowering of blood sugar causes a decrease in phosphorus excretion.

In all of these experiments there was a decrease in serum inorganic phosphate after either the oral or intravenous administra-

TABLE III

Phosphorus Content of Swine Organs during Absorption

The values are given in mg. per cent.

		Duodenal mucosa		Liver		Kidney	
		Inorganic P	Ester P	Inorganic P	Ester P	Inorganic P	Ester P
Fasting	Maximum	225	527	280	477	243	383
	Minimum	205	487	215	409	217	347
	Mean	215	509	250	439	231	368
1 hr. after glucose	Maximum	195	645	242	454	239	497
	Minimum	188	560	212	358	221	378
	Mean	190	612	225	400	229	422
2 hrs. after oil	Maximum	291	823	272	563	255	604
	Minimum	244	737	252	536	198	486
	Mean	271	768	262	549	219	526

tion of glucose.   This is in complete agreement with the literature (21) and the accepted belief.

Analyses of swine organs after oil ingestion were made in an effort to find in which organs the phosphorus is mobilized.   A few attempts to catheterize sows after food ingestion were made but it was decided that the struggles of the animals would have a greater effect on the values than the food.

A study of Table III shows that after oil ingestion there is a marked increase in the phosphorus of the duodenal mucosa, liver, and kidney.   These increases are mainly in the ester fraction, an increase in the inorganic fraction being significant in the mucosa

only. The function of these increases is conjectural. Borri (22) has also reported an increase in soluble phorphorus of the intestinal mucosa during oil absorption and attributed it to a breakdown in "lecithalbumin" to supply glycerol for the resynthesis of triglycerides. Laszt and Sullmann (23) obtained conflicting results in testing for an increase in acid-soluble phosphorus in the intestinal mucosa of rats absorbing olive oil.

Since the liver is said to play an important rôle in the intermediary metabolism of fat, and the phosphorylation of the fat is a possible stage in that metabolism, the increase in liver phosphate during fat absorption is a logical finding. The explanation for the increase in kidney phosphate is not so apparent.

In Table III are also presented the phosphate changes in swine organs after the ingestion of glucose. The decrease in the inorganic fraction and increase in the ester fraction in the intestinal mucosa are similar to the results recorded by Laszt and Sullmann (23) except that these authors found no increase in total acid-soluble phosphorus, the increase in the ester fraction being equal to the decrease in the inorganic fraction.

There is no significant change in phosphate fractions of the liver. Cori and Goltz (24) found an increase in inorganic phosphorus in the liver after insulin injection but no evidence of increased hexose phosphate formation. As pointed out above, it should not be assumed that the injection of insulin and the administration of glucose have the same effect on the mobilization of phosphates.

Fenn (25) found a decrease in the inorganic and an increase in the ester phosphates of the liver during the active assimilation of starch and sucrose, while Takahisa (26) found an increase in the inorganic phosphate of rabbit livers after the oral administration of glucose.

There is no change in the inorganic phosphate of the kidney during glucose absorption but there is a possible increase in the ester fraction. The function of this additional phosphate may be the increased resorption of glucose from the kidney tubules due to the alimentary hyperglycemia.

There was an increase in dry substance in the intestinal mucosa during absorption. This was especially significant after glucose ingestion and might be due to simple osmosis as a result of the high concentration of the sugar in the intestine. The mean pe.

centage of dry substance in the mucosa was as follows: during fasting 15.9, after oil 17.8, after glucose 19.3.

SUMMARY

1. There is a decrease in serum and urinary inorganic phosphate after the ingestion of olive oil by humans.

2. There is a decrease in serum but an increase in urinary phosphorus after the oral or intravenous administration of glucose to humans.

3. During the absorption of glucose by swine there is (a) a decrease in the inorganic and an increase in the ester phosphorus of the duodenal mucosa, (b) no significant change in the phosphate fractions of the liver, and (c) no change in the inorganic but an increase in the ester phosphate of the kidney.

4. During the absorption of cottonseed oil by swine there is (a) an increase in both phosphate fractions of the duodenal mucosa, (b) no change in the inorganic but an increase in the ester phosphate of the liver, and (c) no change in inorganic but an increase in ester phosphate of the kidney.

5. There is an increase in dry substance in the duodenal mucosa of swine during the absorption of glucose.

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THE COMPLEX NATURE OF THE ALCOHOL PRECIPITATE FACTOR REQUIRED BY THE CHICK

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Bauernfeind, Schumacher, Hodson, Norris, and Heuser (1), Bauernfeind and Norris (2), and Schumacher and Heuser (3, 4) reported that chicks and hens require a previously unrecognized factor, or factors, for normal growth and reproduction. This factor was termed the alcohol precipitate factor, since it is precipitated from a concentrated water extract of dried brewers' yeast upon the addition of 10 volumes of 95 per cent alcohol. In further research work the addition of this factor to special basal Diet 540, developed by Schumacher and Heuser (4), promoted growth in white Leghorn chicks which was approximately equal to that obtained with dried brewers' yeast. The average weight of these groups of chicks at 6 weeks of age was 373.0 and 377.4 gm. respectively, while that of the control group was 195.4 gm. These results showed that the alcohol precipitate factor obtained from a water extract of yeast was qualitatively equal in growth-promoting effect to the untreated yeast, when added to Diet 540. As the work on the alcohol precipitate factor continued, it became evident that it is composed of at least two factors required for chick growth rather than one. It is the purpose of this report to present results which demonstrate the existence of these two factors and show their non-identity with the vitamins generally accepted as being required by the chick.

EXPERIMENTAL

Single comb white Leghorn chicks were used in all of the experiments. Ten to twenty chicks were used per pen. Some experiments included both males and females, while in others only males were used. When both sexes were included, the

average weight was weighted for sex. All of the chicks were placed on the basal diet for a depletion period of 7 days, in order to obtain those possessing a more uniform storage of the factor by eliminating the most rapidly and slowest growing chicks. In general, only 50 to 60 per cent of the original number of chicks placed on the basal diet was used in the experiments. All experiments were conducted for a period of 6 weeks.

In these studies chick basal Diet 540 was used. The composition of this diet is given in Table I. The supplements were mixed into the basal diet. The composition of the diet was changed very little by the addition of dry supplements, since large amounts were not used. When liquid supplements were

TABLE I

Chick Basal Diet 540

Ingredient	Per cent
Degerminated corn-meal...........................	60.5
Peanut meal.......................................	15.0
Purified casein....................................	11.0
Cane molasses.....................................	5.0
Salt Mixture 101 (4)..............................	5.0
Soy bean oil......................................	3.0
Cod liver oil.....................................	0.5
	γ *per 100 gm.*
Riboflavin..	400
Thiamine...	200

added, the mixture was dried in a wind tunnel before feeding. The composition of the salt mixture used in the basal diet was previously reported by Schumacher and Heuser (4).

Extraction and Separation of Factor R and Factor S—These two factors were prepared as follows: 5000 gm. of dried brewers' yeast were extracted with 20 liters of 0.24 N HCl solution for 1 hour at 100°, with constant stirring. After cooling, the material was filtered and the residue again extracted as before. The combined filtrates were concentrated *in vacuo* (660 mm. of Hg) until 1 cc. of the filtrate was equivalent to approximately 2 gm. of the original yeast. 10 volumes of 95 per cent ethyl alcohol were slowly added to the concentrated filtrate with constant

stirring. A precipitate formed. The pH of the solution was 1.5 to 1.8. The mixture was allowed to stand 4 to 6 hours, during which time the precipitate settled. The precipitate was filtered off and thoroughly washed with 95 per cent alcohol. The acid alcohol filtrate was then concentrated *in vacuo* until 1 cc. was equivalent to 4 gm. of yeast.

It will be shown later that the acid alcohol precipitate contains one growth factor and the acid alcohol filtrate contains another growth factor. In this paper the factor remaining in the acid alcohol filtrate is referred to as factor R, while the factor precipitated by the acid alcohol treatment is referred to as factor S.

Experiments were conducted in which graded amounts of the acid alcohol filtrate and the acid alcohol precipitate were fed as well as combinations of these fractions.

In Experiments 1 and 2 (Table II) graded amounts of the alcohol filtrate factor were fed. Chicks fed the basal diet alone in Experiment 1 averaged 222.0 gm. at 6 weeks of age. Those receiving the basal diet plus 5 per cent of dried brewers' yeast averaged 383.5 gm. When factor R (acid alcohol filtrate) was fed at levels equivalent to 3, 6, and 9 per cent of yeast, the average weight of the chicks in each pen was approximately 300 gm. This showed that even when the quantity of supplement was increased by 3 times the original amount, a plateau was reached at a subnormal growth response rather than progressive growth increases experienced. However, when factor S (acid alcohol precipitate), equivalent to 5 per cent of yeast, was added to the basal diet containing factor R, equivalent to the same amount of yeast, growth equaled that obtained with 5 per cent of yeast.

In Experiment 2 a similar assay was conducted except that factor R was further purified by precipitating it from the acid alcohol solution by neutralization. When this neutral alcohol precipitate was fed at levels equivalent to 5, 10, and 15 per cent of yeast, the average weight of chicks in each pen was again approximately 300 gm. at 6 weeks of age as compared to 232.3 gm. for chicks on the basal diet. When factor S (acid alcohol precipitate), equivalent to 5 per cent of yeast, was added to the basal diet containing factor R, equivalent to the same amount of yeast, growth was superior to that obtained with factor R alone and equal to that obtained with 5 per cent of yeast.

These results demonstrated that equal, but subnormal, growth response is obtained by feeding graded amounts of factor R and that the original alcohol precipitate factor is a complex composed of at least two factors.

TABLE II

Equal, but Subnormal, Growth Response Obtained with Graded Amounts of Factor R

Treatment	Average weight at 6 wks.	
	Experiment 1	Experiment 2
	gm.	*gm.*
Basal Diet 540.............................	222.0	232.3
" diet + factor R ≈ 5% yeast*...........	294.3	301.4
" " + " " ≈ 10% " *...........	296.5	301.0
" " + " " ≈ 15% " *...........	298.4	303.5
" " + " " + factor S ≈ 5% yeast..	375.0	375.5
" " + 5% yeast......................	383.5	374.0

* The graded amounts of factor R fed in Experiment 1 were equivalent to 3, 6, and 9 per cent of yeast.

TABLE III

Equal, but Subnormal, Growth Response Obtained with Graded Amounts of Factor S

Treatment	Average weight at 6 wks.	
	Experiment 3	Experiment 4
	gm.	*gm.*
Basal Diet 540.............................	195.4	242.4
" diet + factor S ≈ 5% yeast...........	232.2	308.5
" " + " " ≈ 10% "	241.6	316.2
" " + " " ≈ 15% "	234.7	313.3
" " + " " + factor R ≈ 5% yeast..	375.0	391.0
" " + 5% yeast......................	377.4	398.1

In Experiments 3 and 4 (Table III) graded amounts of factor S were fed. The chicks on the basal diet averaged 195.4 and 242.4 gm. respectively. When the basal diet was supplemented with factor S (acid alcohol precipitate), equivalent to 5, 10, and 15 per cent of yeast, a plateau was likewise reached at a subnormal growth response. When factor R (acid alcohol filtrate), equiva-

lent to 5 per cent of yeast, was added to a diet containing factor S, equivalent to the same amount of yeast, growth was increased to the more normal response obtained with 5 per cent of yeast. Again it was shown that the basal diet used in these studies is deficient in two factors; namely, factor R which is contained in the alcohol filtrate fraction and factor S which is present in the alcohol precipitate fraction.

A general summary of these experiments is presented in Fig. 1. It will be noted that chicks fed the basal diet alone averaged approximately 225 gm. at 6 weeks of age. When this basal diet was supplemented with graded amounts of factor S (acid alcohol
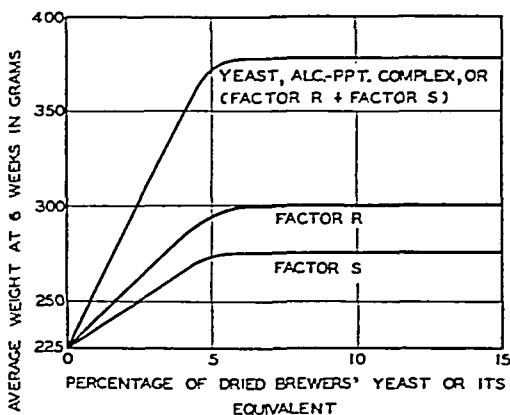
FIG. 1. The plateau of the factor R and factor S growth response curves at subnormal levels.

precipitate) only, growth was increased to approximately 275 gm. When the basal diet was supplemented with factor R (acid alcohol filtrate) only, growth was increased to approximately 300 gm. Regardless of the amount of each of the factors added, a plateau was reached at a level markedly above the growth response obtained with the basal diet but subnormal to that obtained when yeast was added to the basal diet. When the two factors were combined, growth was equal to that obtained with 5 per cent of dried brewers' yeast or the alcohol precipitate factor complex equivalent to this amount of yeast. A statistical analysis of the results showed that the growth differences between the average

weights of the chicks on the basal diet and of those receiving factor R (or factor S) and the differences between the average weights of chicks receiving factor R (or factor S) and of those receiving factor R plus factor S were highly significant statistically, as the odds in all cases were greater than 999:1.

<div align="center">DISCUSSION</div>

The growth factors reported in these experiments are not identical with the recognized water-soluble vitamins or factors. Synthetic riboflavin and thiamine were added to the basal diet in amounts exceeding those required for normal growth. Unpublished data from this laboratory showed that nicotinic acid is not identical with either of these factors, since the addition of this vitamin to the basal diet had no effect on growth. Mickelsen, Waisman, and Elvehjem (5) also reported that nicotinic acid did not cure chick dermatitis or influence growth. The peanut oil meal and cane molasses included in the basal diet supplied adequate amounts of pantothenic acid according to the requirement as reported by Jukes and Lepkovsky (6). Furthermore, no indication of a deficiency of pantothenic acid was ever observed in chicks fed the basal diet, and concentrates of the alcohol precipitate factor complex when fed to chicks on a heated diet[1] produced lesions of dermatosis equal to those obtained with the heated diet alone. Microbiological assays[1] showed these concentrates to be nearly free of pantothenic acid and the basal diet to contain at least 50 per cent more[1] than is required by the chick. Neither one of the factors is identical with pyridoxine (vitamin B6) since in several experiments, when this vitamin was fed at a level of 300 γ per 100 gm. of diet, no increase in growth was obtained over that on the basal diet alone.

These factors are not identical with the chondroitinsulfuric acid factor, reported by Robinson, Gray, Chesley, and Crandall (7), since the addition of chondroitinsulfuric acid at a 2 per cent level to the basal diet used in these studies did not promote any significant increase in growth over that obtained on the basal diet alone. The presence of factors R and S in yeast makes it improbable that either one is identical with the grass juice factor

[1] Unpublished results of J. C. Bauernfeind, Department of Poultry Husbandry, Cornell University.

of Kohler, Elvehjem, and Hart (8), as they have shown that yeast contains little of this factor. Differences in solubility point to the non-identity of these factors with factor W as reported by Frost and Elvehjem (9). It is also improbable that either factor is identical with the factor reported by Stokstad and Manning (10) in polished rice, since the amounts of corn-meal and peanut meal in basal Diet 540 are considerably above the amounts which they indicate as being necessary to promote growth and livability of chicks.

Since the factors herein reported are growth-stimulating and have not produced any macroscopic symptoms, they are not identical with those factors for which specific effects have been reported. Hegsted, Oleson, Elvehjem, and Hart (11) concluded that chicks require a factor, present in cartilage, kidneys, and rice, a lack of which produced a dermatitis but apparently did not influence growth. Jukes and Babcock (12) indicated the necessity of an antiparalytic factor in the chick diet.

The only factor that has any similarity to the factors reported here is factor U of Stokstad and Manning (13). Recently Stokstad, Manning, and Rogers (14) reported factor U to be a complex consisting of pyridoxine and some other factor for which the term factor U is retained. Whether or not either of the factors reported herein is the same as factor U must await further study.

SUMMARY

Evidence has been presented showing that chicks require two dietary growth factors not identical with the following known vitamins or factors: vitamin A, vitamin D, α-tocopherol, vitamin K, thiamine, pyridoxine, riboflavin, pantothenic acid, nicotinic acid, the grass juice factor, factor W, the antiencephalomalacia factor, the chondroitinsulfuric acid growth factor, the cartilage growth factor, the antiparalytic factor of Jukes, and the polished rice factor reported by Stokstad and Manning. Whether or not either one of the factors reported in this paper is identical with factor U cannot be stated at the present time.

These two growth factors are found in large amounts in dried brewers' yeast. The factors are extracted from yeast with 0.24 N HCl solution and separated by alcohol precipitation upon adjust-

ment of the pH. Factor R is soluble in acid alcohol, while factor S is precipitated in this solution. Factor R can be further purified by neutralization of the acid alcohol filtrate, by which procedure the factor is then obtained as a neutral alcohol precipitate.

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SYNTHESIS OF THE $d(+)$-α-GLYCEROPHOSPHORIC ACID AND THE ACTION OF PHOSPHATASES ON SYNTHETIC $d(+)$-, $l(-)$-, AND dl-α-GLYCEROPHOSPHORIC ACIDS*

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The synthesis of the racemic α-glycerophosphoric acid (1) and of the $l(-)$-α-glycerophosphoric acid (2) has been reported. The $l(-)$-α-glycerophosphoric acid has been isolated from various natural sources, such as phosphatides, and as an intermediate product of fermentation or glycolysis. For this reason, in a previous paper we called the $l(-)$-α-glycerophosphoric acid the "biological" acid. The $d(+)$-α-glycerophosphoric acid has not yet been found in nature. We have carried out its synthesis in the same way as that of its optical antipode, and describe in this paper its preparation in the form of the barium salt and silver salt. The diethyl ether diethyl ester possessed in homogeneous substance the rotation $[\alpha]_D^{20} = +5.94°$; in absolute ethyl alcohol $[\alpha]_D = +6.69°$. The principle of the synthesis, in which $l(-)$-acetone glycerol (3) is used as starting material is given in the accompanying formula which also explains the configurational relationship between $d(+)$-α-glycerophosphoric acid and l-glyceraldehyde.

This method of synthesis of the α-glycerophosphoric acids not only gives products of known configuration, but also produces the enantiomorphs in optically pure form, thus having an obvious advantage over previous methods (5, 6) which relied on resolution of the racemic acid.

Since in many cases the deciding factor for the biological utilizability of substances with an asymmetrical carbon atom, such as

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* This paper is Communication VIII of the series, "Studies on acetoneglyceraldehyde, and optically active glycerides."

Acetone *l*-glyceraldehyde   *l* (−)-Acetone glycerol

$$\xrightarrow[\text{quinoline}]{POCl_3}$$

$$\longrightarrow \quad \begin{array}{c} H_2C \cdot O \cdot PO_3Ba \\ | \\ HO \cdot {}^*C \cdot H \\ | \\ HO \cdot CH_2 \end{array}$$
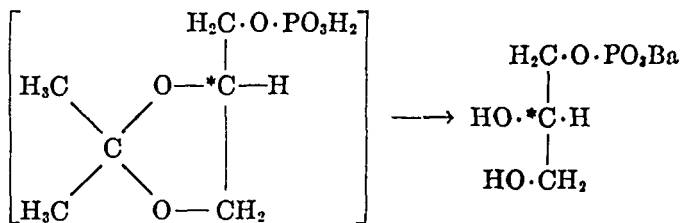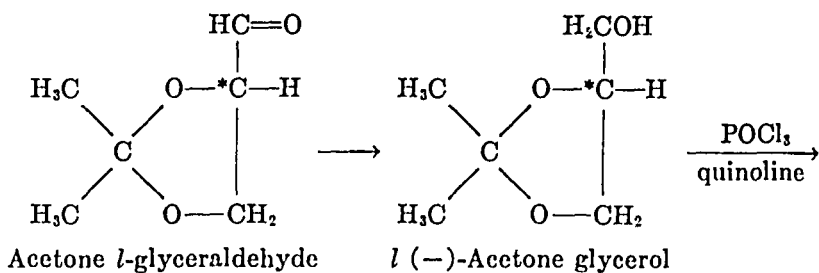
Barium *d*(+)-α-glycerophosphate*

* For the configuration and nomenclature see our earlier papers (2, 4).

sugars and amino acids, seems to be their configuration, we are inclined to believe that also in the field of naturally occurring glycerol derivatives[1] the asymmetry of the β-carbon atom of the glycerol should play a similar rôle.

In an attempt to establish the significance of that asymmetry, we began the investigation of the action of enzymes on the optical isomers of various glycerol derivatives, such as mono-, di-, and triglycerides, glycerol ethers, glycerol acetals with fatty acid residues, and glycerophosphates. A study of the action of phosphatases on glycerophosphates was undertaken first because the glycerophosphates are less liable to acyl migration than glycerides with fatty acid residues and because it was already known that the α-glycerophosphates react differently with the ferments of muscle press-juice. Meyerhof and Kiessling (7) found, using the racemic compound as substrate, that only the *l*(−)-α-glycerophosphoric acid was used up completely, the *d*(+)-α-glycerophosphoric acid remaining unchanged.

Experiments repeated recently by Professor O. Meyerhof[2]

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[1] In regard to the great number of naturally occurring mixed acid triglycerides, this asymmetry of the β-carbon atom of the glycerol part of the molecule may in the future be found to have biological significance.

[2] Meyerhof, O., private communication.

in Paris with samples of pure $l(-)$- and $d(+)$-α-glycerophosphoric acid provided by us have confirmed the previous result obtained.

The rate of hydrolysis of $l(-)$-α-glycerophosphoric acid and $d(+)$-α-glycerophosphoric acid by unrefined kidney phosphatase, taka-phosphatase, rat bone phosphatase, and a purified phosphatase from dog feces has been investigated by us. We were able to show with all four preparations that, under the conditions specified, the $d(+)$-α-glycerophosphoric acid was hydrolyzed with greater velocity than the $l(-)$-α-glycerophosphoric acid. This fact has probably some physiological significance. We were interested primarily in the synthesis of the α-glycerophosphoric acids with definite configuration. The behavior of the optical isomers toward ferments was investigated to prove the biological significance of the asymmetry of the β-carbon atom in glycerol derivatives. Having established this point, we do not propose to extend the enzyme work on glycerophosphates any further.

In the experimental part of this paper details are given for the hydrolysis with two enzymes only; namely, kidney phosphatase with ammonium buffer (pH 9.6) and phosphatase from dog feces with carbonate-veronal buffer (pH 8.62). The method used to follow the rate of hydrolysis was the same as that described by King and Armstrong (8), and the determination of free phosphoric acid was carried out as applied by King (9). Data showing the rate of hydrolysis of the two optical isomers are given in Tables I and II and Figs. 1 and 2, and in Fig. 2 a curve for the racemic compound is included. The racemic compound is hydrolyzed, as was to be expected, at a rate approximately midway between those of its two components. Taka-phosphatase with phthalate buffer (pH 3.8) and rat bone enzyme with carbonate-veronal buffer (pH 8.62) gave essentially similar results.

Conclusive proof, however, that the configuration of asymmetrically substituted glycerols has an important bearing on their physiological activity will require extension of the present research to other types of glycerol derivatives. In this connection we are at present studying the enzymatic hydrolysis of the optically active glycerides with fatty acid residues and hope to present our results in the near future.

We would like to thank Dr. E. J. King, British Post-Graduate Medical School, London, England, for having kindly put at our disposal his veronal-CO_2 buffer (10) before publication. We
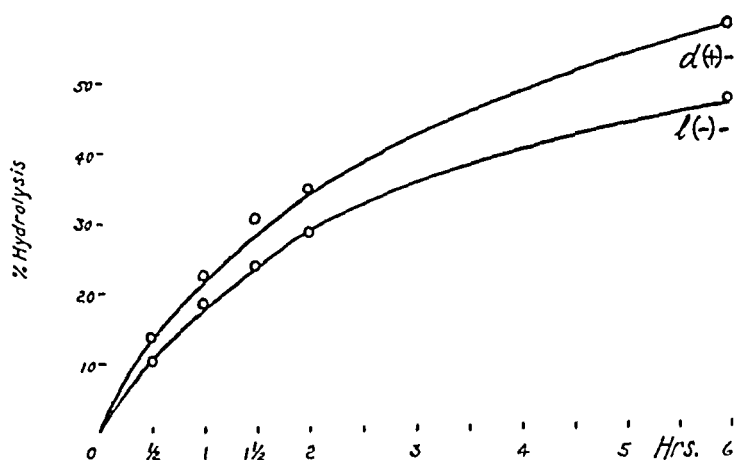
FIG. 1. Hydrolysis of $l(-)$- and $d(+)$-α-glycerophosphate by pig kidney phosphatase.
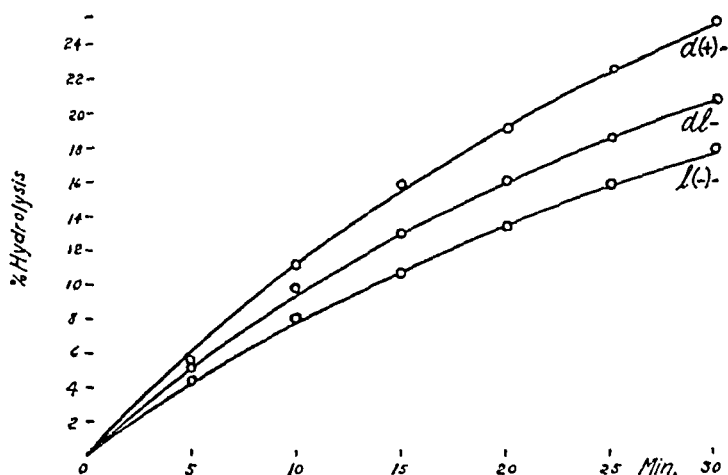
FIG. 2. Hydrolysis of inactive, $l(-)$-, and $d(+)$-α-glycerophosphate by phosphatase prepared from dog feces.

also wish to thank him and Dr. C. C. Lucas of the Department of Medical Research, Banting Institute, University of Toronto, for their kind advice and assistance during our enzyme experiments.

Our thanks are also due to Miss Shirley Platt for the care with which she conducted the enzyme experiments.

TABLE I

Hydrolysis of l(−)- and d(+)-α-Glycerophosphate by Pig Kidney Phosphatase

8 cc. of ammonium buffer (pH 9.6), 8 cc. of substrate (containing 8.0 mg. of organic P), and 4 cc. of enzyme solution were used.

Time	Phosphorus hydrolyzed (corrected for blank)	
	l(−)-	d(+)-
	mg.	mg.
0 min.	0.0	0.0
30 "	0.840	1.107
60 "	1.480	1.834
90 "	1.918	2.464
120 "	2.264	2.785
6 hrs.	3.751	4.567

TABLE II

Hydrolysis of Inactive, l(−)-, and d(+)-α-Glycerophosphate by Phosphatase Prepared from Dog Feces

25 cc. of carbonate-veronal buffer (pH 8.62), 20 cc. of substrate (containing 6.2 mg. of organic P), and 5 cc. of enzyme solution were used.

Time	Phosphorus hydrolyzed (corrected for blank)		
	l(−)-	dl-	d(+)-
min.	mg.	mg.	mg.
0	0.0	0.0	0.0
5	0.275	0.333	0.350
10	0.500	0.617	0.700
15	0.667	0.817	0.983
20	0.833	1.00	1.183
25	0.983	1.150	1.397
30	1.100	1.283	1.567

EXPERIMENTAL

Synthesis of Substrate

Barium Salt of d(+)-α-Glycerophosphoric Acid (Prepared from l(−)-Acetone Glycerol)—The method used for the preparation of d(+)-α-glycerophosphoric acid is the same as that given by us

for the simplified preparation of *l(−)-α*-glycerophosphoric acid (2). 4.1 gm. of *l(−)*-acetone glycerol (3) yielded 3.7 gm. of barium salt (37 per cent of the theoretical).

$C_3H_7O_6PBa$ (307.48).   Calculated.   C 11.7, H 2.3, P 10.08
                     Found.   " 11.7, " 2.6, " 10.04

Diethyl Ether of Ethyl d(+)-α-Glycerophosphate—

$$
\begin{array}{c}
OC_2H_5 \\
\diagup \\
H_2C\cdot O\cdot P\!=\!O \\
| \qquad\qquad \diagdown \\
H_5C_2\cdot O\cdot{}^*C\cdot H \qquad OC_2H_5 \\
| \\
H_2C\cdot O\cdot C_2H_5
\end{array}
$$
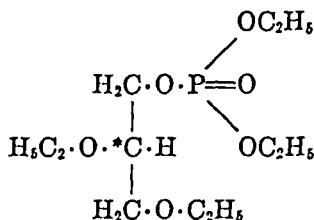
The diethyl ether of ethyl *d(+)-α*-glycerophosphate was prepared according to the prescription given for its antipode, diethyl ether of ethyl *l(−)-α*-glycerophosphate.

Silver d(+)-α-Glycerophosphate—2.4 gm. of barium *d(+)-α*-glycerophosphate gave 2.42 gm. of silver salt (80 per cent of the theoretical).
     $C_3H_7O_6PAg_2$ (385.8).   Calculated, Ag 56.4; found, Ag 56.3

Ethylation—2.1 gm. of silver salt yielded 1.2 gm. of diethyl ether of ethyl *d(+)-α*-glycerophosphate (77.6 per cent of the theoretical); b.p. (0.09 mm.) = 95–97°, b.p. (0.22 mm.) = 104–105°, $n_D^{19} = 1.4252$, $n_D^{23} = 1.4238$.
     $C_{11}H_{25}O_6P$ (284.2).   Calculated.   C 46.4, H 8.8, P 11.00
                         Found.   " 45.3,* " 8.6, " 10.88

Optical Rotation—(1) In homogeneous substance in a 1 dm. tube, $d_4^{21} = 1.090$, $\alpha_D^{20} = +6.48°$, $[\alpha]_D^{20} = +5.94°$; (2) in absolute ethyl alcohol (distilled over sodium), 0.3403 gm. in 2 cc., *c* = 14.01, 1 dm. tube, $\alpha_D = +0.94°$, $[\alpha]_D = +6.69°$.

* See also Paper VI (2), especially p. 499, for the corresponding deficiency of carbon in the analysis of the isomer.

The respective rotations of the diethyl ether of ethyl *l(−)-α*-glycerophosphate were (1) in homogeneous substance $[\alpha]_D^{19} = -5.31°$, (2) in absolute ethyl alcohol $[\alpha]_D^{19} = -5.76°$. The optical rotation of the diethyl ethers of diethyl *d(+)*- and *l(−)-α*-glycerophosphates is strongly dependent on the water content of the alcohol used as solvent.

Enzymatic Studies

Substrate Solutions—The action of phosphatases on the *α*-glycerophosphoric acids was studied with the sodium salts of

$l(-)$-, $d(+)$-, and dl-α-glycerophosphoric acids. These were prepared from the corresponding barium salts (1, 2) by treatment with sodium sulfate. The solutions of the sodium salts were kept in the ice box and never used when more than 1 week old.

Preparation of Enzymes. Phosphatase from Kidney—The phosphatase solution from fresh pig kidney was prepared in the usual manner (11) by grinding the tissue with sand and extracting the enzyme with chloroform water. The enzyme solution finally obtained was brought to pH 9.0.

Phosphatase from Dog Feces—According to Armstrong's prescription (12), 0.34 gm. of an active phosphatase preparation (activity about 25,000 units per gm.) was obtained from 1 kilo of dog feces. Standard enzyme solutions were prepared by dissolving approximately 10 mg. of this solid material in 25 cc. of physiological saline. This stock solution was diluted to give enzyme solutions of a suitable activity (ranging from 0.1 to 0.6 unit per cc. (8)).

Buffer Solutions. Ammonia Buffer—An ammonia buffer of pH 9.6 was prepared by adding 1 volume of 0.2 M NH_4Cl to 6 volumes of 0.2 M NH_4OH.

Carbonate-Veronal Buffer—A carbonate-veronal buffer of pH 8.62 (at 37°) was prepared according to King and Delory (10).

Rate of Hydrolysis with Kidney Phosphatase—Ammonia buffer, enzyme, and substrate solutions were separately brought to 37.5°. Then 8 cc. of buffer and 8 cc. of substrate solution (0.033 M), followed by 4 cc. of enzyme solution were thoroughly mixed in a large test-tube and kept at 37.5° in a thermostat with occasional stirring. Aliquots (3 cc.) were removed at zero time, 30 minutes, 60 minutes, $1\frac{1}{2}$ hours, 2 hours, and 6 hours, and immediately run into 2 cc. of trichloroacetic acid to inactivate the enzyme. The phosphoric acid liberated was determined colorimetrically by the method described by King (9). The inorganic phosphate found in the zero time aliquot was taken as representing the sum of the free phosphate in the enzyme and substrate used. The figures obtained are corrected for this "blank" value and reported in Table I and Fig. 1. Similar results were obtained with other concentrations of the substrate solution.

Rate of Hydrolysis with Phosphatase from Dog Feces—The experiments with the enzyme from dog feces were conducted as described above with the following changes: (1) the aforementioned carbonate-veronal buffer (pH 8.62) was used; (2) a weaker

substrate solution was used (0.010 M) and a shorter period of time was allowed for the hydrolysis, as the enzyme was so much more active than the kidney preparation; (3) the use of trichloroacetic acid to precipitate protein was unnecessary because of the purified nature of the enzyme. In this case the hydrolysis was stopped by running the aliquots into the 1 cc. of perchloric acid used for the colorimetric phosphate determination.

The data for these hydrolyses are given in Tables I and II and Figs. 1 and 2.

SUMMARY

1. With *l*(−)-acetone glycerol as starting material the *d*(+)-α-glycerophosphoric acid has been prepared.

2. In experiments on the action of various phosphatases on *l*(−)-α-glycerophosphoric acid and *d*(+)-α-glycerophosphoric acid, the *d*(+)-α-glycerophosphoric acid was hydrolyzed with markedly greater velocity than the *l*(−)-α-glycerophosphoric acid, while the rate of hydrolysis of the racemic compound was about midway between the rates of the other two. In contrast, muscle press-juice has been observed by Meyerhof to utilize *l*(−)-α-glycerophosphoric acid completely, while it does not utilize *d*(+)-α-glycerophosphoric acid at all.

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THE EFFECT OF CHOLINE ON THE BLOOD AND LIVER LIPIDS OF THE DOG SUBJECTED TO LIGATION OF THE PANCREATIC DUCTS*

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I. L. CHAIKOFF

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Berkeley, and the Division of Surgery, University of California
Medical School, San Francisco)

Recent studies on the dog subjected to ligation of the pancreatic ducts suggest an important parallelism between the animal so prepared and the completely depancreatized dog maintained with insulin. Two points of similarity have been observed: (1) when maintained on a diet adequate in calories, proteins, vitamins, and salts but lacking raw pancreas, both develop fatty livers and show a fall in the level of all lipid constituents of the blood (1–4); (2) in both types of animals the ingestion of raw pancreas prevents the deposition of excessive amounts of fat in the liver and maintains the lipids of the blood at or above normal levels (2, 5). In view of the physiological importance of these findings, it seemed desirable to extend the comparison of the two methods of preparation. In the present study the effects of ingested choline upon the blood and liver lipids of the duct-ligated dog are determined and compared with previous findings in which it was shown that, in the depancreatized dog kept alive with insulin, choline, administered orally, prevents the infiltration of fat in the liver and maintains the blood lipids at about normal levels (6).

EXPERIMENTAL

Twelve dogs were subjected to ligation of the pancreatic ducts. Four were used for controls; eight were fed 2 gm. of choline chloride

daily. With one exception, all animals were sacrificed 19.5 to 31 weeks following the commencement of the specific procedure.

Before ligation of the pancreatic ducts, the dogs were maintained for 1 to 4 weeks on a diet of meat, sucrose, and vitamin supplements. Each animal received daily 30 gm. of lean meat per kilo of body weight, 6 gm. of sucrose per kilo, 5 gm. of bone ash, and 2 gm. of Cowgill's salt mixture (7). Vitamins A and D were furnished in the form of a standardized cod liver oil; the B complex as a concentrate prepared from rice bran.[1]

The method employed for ligation of the pancreatic ducts, as well as the precautions found necessary to insure their complete occlusion, has been recorded elsewhere (1). Following division of the ducts, each animal received twice daily a diet consisting of 250 gm. of lean meat and 25 gm. of sucrose, as well as bone ash and the vitamin supplements. In view of the impaired absorption that follows exclusion of the pancreatic juice from the intestinal tract, no attempt was made to adjust the diet on the basis of body weight. Food was administered at 8.00 a.m. and 4.00 p.m. daily. With each meal the dogs received 8 units of insulin[2] subcutaneously. This precaution was taken to make sure of an adequate supply of insulin in the presence of acinous and possible islet tissue atrophy (1).

The ingestion of choline by the eight dogs was not started until a satisfactory appetite had been established. As a rule the animals maintained a vigorous appetite, but on occasions it was found necessary to resort to forced feeding to insure complete ingestion of the prescribed diet. During the interval (which in some cases extended to as long as 4 weeks) between the operation and the commencement of the daily administration of choline, each dog received 125 gm. of raw pancreas twice daily in addition to the standard diet. This precaution was believed to insure the existence of a normal liver at the time the choline feeding was initiated. Two dogs (Dogs E-39 and E-41) showed no postoperative depres-

sion of appetite. They were started on the choline therapy 24 hours after ligation of the pancreatic ducts.

At the time blood and liver were removed for analyses, all dogs were in the postabsorptive state, having ingested their last meal approximately 16 hours previously. Whole blood was used for lipid analyses. The liver was removed after the animal had been anesthetized with nembutal. A mixed sample of the *whole* liver was taken for lipid estimations. The oxidative procedures em-

TABLE I

Effect of Choline on Liver Lipids of Duct-Ligated Dog

The values are expressed as per cent of wet tissue.

Dog No.	Weight			Interval since duct ligation	Period of raw pancreas administration	Period of choline administration	Choline chloride fed per day	Liver weight	Cholesterol			Total fatty acids	Phospholipid	Residual fatty acids
	Preoperative	When choline added	Final						Total	Free	Ester			
	kg.	kg.	kg.	wks.	wks.	wks.	gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent
E-38	15.0		10.1	22.5			0	425	0.48	0.23	0.25	14.80	1.89	13.40
E-47	21.5		12.0	20.0			0	493	0.54	0.22	0.32	25.70	1.68	24.50
E-48	13.4		10.1	20.0			0	460	0.31	0.16	0.15	10.30	1.38	9.30
E-55	9.6		7.9	20.0			0	460	0.40	0.31	0.09	7.60	2.17	6.10
E-39	11.1	11.1	8.4	23.3	0	23.3	2	250	0.25	0.24	0.01	3.86	3.10	1.60
E-41	16.5	16.5	9.0	7.0	0	7.0	2	315	0.29	0.22	0.07	2.46	2.69	0.46
E-43	13.3	13.0	8.0	30.0	3.5	26.5	2	330	0.30	0.12	0.18	2.33	1.82	1.00
E-46	12.5	13.3	10.4	35.0	3.5	31.5	2	260	0.15	0.16		1.91	1.81	0.61
E-50	13.5	13.1	11.0	24.3	4.0	23.0	2	230	0.30	0.27	0.03	3.90	2.57	2.17
E-51	19.3	15.2	12.9	24.3	4.0	23.0	2	380	0.29	0.20	0.09	3.16	2.05	1.76
E-53	12.2	12.9	8.7	24.3	4.0	23.0	2	270	0.27	0.23	0.04	2.27	2.56	0.52
E-54	10.3	11.0	8.0	23.0	3.0	20.0	2	215	0.32	0.25	0.07	2.78	1.80	1.68

ployed for lipid analyses of blood and liver have been described elsewhere (1, 2).

Results

Liver Lipids—The fatty acids, phospholipid, and free and esterified cholesterol contents of the liver of the twelve dogs are shown in Table I. The four control animals were sacrificed 20 to 22.5 weeks after ligation; their livers contained 7.6, 10.3, 14.8,

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E-55	9.6		7.9	20.0			0	460	0.40	0.31	0.09	7.60	2.17	6.10
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E-41	16.5	16.5	9.0	7.0	0	7.0	2	315	0.29	0.22	0.07	2.46	2.69	0.46
E-43	13.3	13.0	8.0	30.0	3.5	26.5	2	330	0.30	0.12	0.18	2.33	1.82	1.00
E-46	12.5	13.3	10.4	35.0	3.5	31.5	2	260	0.15	0.16		1.91	1.81	0.61
E-50	13.5	13.1	11.0	24.3	4.0	23.0	2	230	0.30	0.27	0.03	3.90	2.57	2.17
E-51	19.3	15.2	12.9	24.3	4.0	23.0	2	380	0.29	0.20	0.09	3.16	2.05	1.76
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and 25.7 per cent fatty acids. Seven dogs were sacrificed at intervals of 20 to 31.5 weeks after the commencement of choline feedings. Their livers contained 1.9 to 3.9 per cent fatty acids. One animal (Dog E-41), 7 weeks after choline treatments were initiated, was sacrificed because of extreme emaciation and weakness. At this time its liver contained 2.5 per cent fatty acids.

<div align="center">TABLE II</div>

Blood Lipids of Duct-Ligated Dogs Maintained on Lean Meat-Sucrose Diet

Dog No.	Period after duct ligation	Weight	Cholesterol			Total fatty acids	Phospholipid	Total lipid	Residual fatty acids
			Total	Free	Ester				
	wks.	kg.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
E-38	(20)*	15.0	161	143	18	366	371	527	87
	(1)	15.0	150	140	10	348	365	498	87
	5	11.3	102	106	0	260	296	362	70
	11.	10.1	78	78	0	200	184	278	54
	18.5	9.8	89	82	7	252	232	341	64
	22.5	10.1	110	92	18	271	262	381	61
E-47	(20)	20.2	204	159	45	466	406	670	96
	(10)	21.0	214	153	61	512	453	726	98
	9.5	18.0	124	118	6	324	279	488	84
	13.5	14.2	120	93	27	202	235	322	36
	20.0	12.0	78	62	16	205	202	283	45
E-48	(10)	12.7	208	135	73	452	410	660	113
	(0)	13.4	197	143	54	492	406	689	88
	9.5	12.7	136	119	17	352	265	488	85
	13.5	11.5	114	114	0	270	269	384	73
	20.0	10.1	72	72	0	262	168	334	71
E-55	(8)	9.4	172	113	59	314	335	486	45
	4	8.4	150	124	26	346	388	496	71
	9.5	8.1	113	109	4	268	312	381	70
	14.0	7.9	104	96	8	288	264	392	73

* The numbers in parentheses refer to the number of days before duct ligation.

Blood Lipids—The effects of choline upon the blood lipid levels of duct-ligated dogs are shown in Tables II and III.   In the former are recorded the blood lipids of four control dogs maintained on a lean meat-sucrose diet for 20 to 22.5 weeks after duct ligation, whereas the dogs shown in Table III received choline in addition to the lean meat-sucrose diet.   In all dogs shown in Table II a

TABLE III

Blood Lipids of Duct-Ligated Dogs Maintained on Lean Meat-Choline Diet

Dog No.	Period after duct ligation	Period of choline administra- tion*	Weight	Cholesterol			Total fatty acids	Phos- pho- lipid	Total lipid	Resid- ual fatty acids
				Total	Free	Ester				
	wks.	*wks.*	*kg.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
E-39	(27)†		10.5	138	120	18	360	305	498	125
	(1)		11.1	164	129	35	354	336	518	86
	5.0	5	9.3	113	104	9	296	364	409	24
	11.0	11	9.0	122	107	15	348	326	470	100
	15.5	15	9.0	126	111	15	348	332	474	96
	19.5	19	8.6	103	103	0	326	351	429	70
E-41	(27)		15.3	199	141	58	429	330	627	149
	(1)		16.5	212	149	63	364	347	576	70
	2.0	2	15.3	141	133	8	336	312	477	103
	5.0	5	11.8	126	123	3	324	296	446	106
	7.0	7	9.0	146	117	29	374	252	520	171
E-43	(10)		13.4	185	131	54	495	337	680	213
	(0)		13.3	190	114	76	471	361	661	157
	5.0	2	12.5	127	112	15	400	350	527	134
	9.0	9	11.2	129	113	16	352	341	481	93
	20.0	20	9.0	140	118	22	344	361	484	66
E-46	(20)		12.0	176	131	45	409	385	585	83
	(10)		12.5	188	137	51	412	420	600	67
	9.0	6	12.2	139	132	7	348	346	487	71
	26.0	23	10.0	111	93	18	328	399	439	26
	31.0	28	10.4	135	93	27	299	343	434	31
E-50	(10)		13.2	175	149	26	427	381	602	131
	(2)		13.5	187	155	32	420	420	607	93
	6.0	2	13.1	193	149	44	460	444	653	107
	11.0	7	12.3	125	94	31	388	367	513	110
	19.0	15	11.5	133	90	43	298	293	431	36
	20.3	16	10.0	143	102	41	323	370	466	26
E-51	(10)		19.2	187	138	49	471	469	658	97
	3.3		15.2	183	155	28	460	384	643	161
	11.0	7	14.0	140	96	44	422		562	
	15.0	11	14.0	119	109	10	302	350	421	99
	20.3	16	12.9	100	82	18	471	341	571	210
E-53	(15)		12.0	152	133	19	362	363	514	91
	(10)		12.2	140	124	16	393	359	533	120
	4.0		12.9	176	136	40	398	411	574	72
	11.0	7	11.2	110	94	16	278	282	388	61
	15.0	11	10.5	120	102	18	306	323	426	57
	23.0	19	8.7	104	106	0	349	369	453	80
E-54	(10)		10.2	153	103	50	320	301	473	67
	7	4	8.2	105	72	33	282	292	388	47
	12	9	8.0	127	109	18	271	361	398	0
	23	20	8.0	121	108	13	315	363	436	51

* Each dog received 2 gm. of choline chloride daily.

† The numbers in parentheses refer to the number of days before duct ligation.

fall in the concentration of all lipid constituents occurred. The most pronounced change was found in cholesterol. This is strikingly demonstrated in Dog E-48, in which total cholesterol fell from a value of 208 to 72 mg. per 100 cc. of whole blood in 20 weeks, while the esterified portion (which was present to the extent of 54 to 77 mg. before duct ligation) completely disappeared as early as 13.5 weeks after the operation. Significant declines in phospholipids and total fatty acids were also observed, while total lipids fell from preoperative levels of 486 to 726 mg. per 100 cc. to 283 to 392 mg. at intervals of 20 to 22.5 weeks after duct ligation.

The feeding of 2 gm. of choline chloride daily failed to maintain the blood lipids of duct-ligated dogs at their preoperative levels (Table III), but in no case did the levels of total lipids in the choline-treated dogs fall as low as those found in animals that received no choline (Table II). Thus, in the untreated dogs, total lipids fluctuated from 283 to 392 mg. per 100 cc. at the end of the periods of observation, whereas at approximately similar intervals after duct ligation the levels of total lipids in the choline-treated dogs fluctuated between 429 and 571 mg. per 100 cc. of whole blood. The final values for total cholesterol were between 72 and 110 mg. per 100 cc. in the control dogs and between 103 and 146 mg. in the choline-fed dogs. Although in no case did the level of total cholesterol fall below 100 mg. per 100 cc. during the entire period in which choline was fed after duct ligation, nevertheless most of these values were below the preoperative ones. Phospholipids reveal a definite response to choline administration when the final values in the choline-treated and non-choline-treated dogs are compared, but here again, when given in doses of 2 gm. daily, choline failed to maintain the concentration of this lipid constituent at its preoperative levels.

DISCUSSION

The development of fatty livers in dogs deprived of the external secretion of the pancreas was indeed striking, but equally so is the present observation that choline completely inhibits the deposition of excessive amounts of fat under identical conditions. Whether this indicates that the pancreatic juice provides a means for supplying choline to the organism cannot be determined from the

present study. The lipid level of the blood, which is depressed by the exclusion of the external secretion of the pancreas, also responds to choline. There is an apparent difference, however, in the response of these two tissues to choline. While 2 gm. of choline chloride held the fat content of the liver at its normal level, this amount failed to restore to normal the concentration of cholesterol, phospholipids, or of total fatty acids of the blood. It should be noted here that all dogs lost weight despite the daily ingestion of 2 gm. of choline chloride, and it is by no means unlikely that the failure of complete restoration of the lipid level to normal in this particular case is associated with the weight loss suffered by the animals. In this connection it has been observed that chronic undernutrition produced in a normal animal by limiting the caloric intake over a period of several months led to a fall in cholesterol, phospholipids, and total fatty acids of the blood (8).

SUMMARY

1. The daily ingestion of 2 gm. of choline chloride completely prevented the appearance of fatty livers in dogs deprived of the external secretion of the pancreas by duct ligation.

2. This treatment failed to maintain the preoperative weights of such animals.

3. The blood lipids of the duct-ligated dog also respond to the continued ingestion of choline, but a dose that kept the lipids of the liver at normal levels was found inadequate for complete maintenance of the blood lipids at preoperative values.

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LETTERS TO THE EDITORS

THE INFLUENCE OF SULFHYDRYL COMPOUNDS UPON THE ACTIVITY OF BONE PHOSPHATASE IN VITRO[*]

Sirs:

The activity of the alkaline phosphatase at optimal pH is inhibited by sulfhydryl compounds;[1] it is restored by iodoacetic acid,[2] but oxidizing agents do not activate the enzyme.[3] The activity of dialyzed extracts of bone phosphatase, prepared by the method of Martland and Robison[4] and tested by the procedure of Bodansky,[5] was studied in the presence of various concentrations of cysteine and other compounds.

Cysteine in low concentration activated the alkaline phosphatase of bone. A maximal activity of the enzyme was observed with an optimal concentration of the cysteine, while with higher concentrations inhibition occurred as shown by Bodansky.[6]

Thioglycolic acid inhibited the phosphatase activity markedly. Most of the activity of the enzyme inhibited by 0.000625 M thioglycolic acid was restored by iodoacetic acid.

Ethyl mercaptan inhibited the enzyme when present in concentrations in excess of 0.0025 M.

Glycine inhibited the phosphatase (when present in a concentration of 0.0250 M) and the enzyme was largely reactivated by iodoacetic acid. The observed reactivation was much greater than the experimental error.

Qualitatively, cysteine and glycine affect the bone phosphatase activity similarly. Cysteine in an optimal concentration gives

[1] Waldschmidt-Leitz, E., Scharikova, A., and Schaffner, A., *Z. physiol. Chem.*, 214, 75 (1932).

[2] Schaffner, A., and Bauer, E., *Z. physiol. Chem.*, 225, 245 (1934).

[3] Pett, L. B., and Wynne, A. M., *Biochem. J.*, 28, 365 (1934). Belfanti, S., Contardi, A., and Ercoli, A., *Biochem. J.*, 29, 517 (1935).

[4] Martland, M., and Robison, R., *Biochem. J.*, 23, 237 (1929).

[5] Bodansky, O., *J. Biol. Chem.*, 118, 341 (1937).

[6] Bodansky, O., *J. Biol. Chem.*, 114, 273 (1936).

Optimal pH, Mg ion 0.0125 M			
Cysteine, M	Activity $Q_{0.05}$	Glycine, M	Activity $Q_{0.05}$
0.025	0.00030	0.063	- 0.00625
0.00063	0.00282	0.025	0.0109
0.000063	0.0100	0.0063	0.0177
0.0000063	0.0216	0.0025	0.0224
0.0000025	0.0220	0.00025	0.0192
0.0000013	0.0186	0.000063	0.0184
0.0	0.0174	0.0	0.0174

Optimal pH, Mg ion 0.0125 M, glycine 0.00625 M			
Thioglycolic acid, M	Activity $Q_{0.05}$	Ethyl mercaptan, M	Activity $Q_{0.05}$
0.0125	0.00039	0.050	0.00053
0.00250	0.00132	0.0125	0.00264
0.000625	0.00700	0.00250	0.00520
0.000125	0.0100	0.00050	0.00607
0.0000250	0.0099	0.00010	0.00647
0.0000063	0.0108	0.000025	0.00617
0.0	0.0105	0.0	0.00623

Optimal pH, Mg ion 0.0125 M			
Glycine, M	Thioglycolic acid, M	Iodoacetic acid, M	Activity $Q_{0.05}$
0.00625			0.0069
0.0250			0.0049
0.0250		0.0250	0.0060
0.00625	0.000625		0.0037
0.00625	0.000625	0.00125	0.0068
0.00625		0.00125	0.0064

the same maximal activity of the phosphatase as does glycine in its optimal concentration. Sulfhydryl compounds inhibit the phosphatase, but apparently not specifically. The reactivation of the inhibited enzyme does not seem to be due to oxidation of the —SH group either of the α-amino acid (cysteine) or of the phosphatase molecule, for the glycine-inhibited enzyme is also reactivated by iodoacetic acid.

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α-TOCOPHERYLQUINONE AND DYSTROPHY IN RABBITS*

Sirs:

Recently it was again demonstrated that α-tocopherylquinone cannot replace tocopherol in curing the sterility of vitamin E-deficient female rats.[1,2] When wheat germ or wheat germ oil was treated with ferric chloride, the oil lost most of its capacity to prevent sterility but retained much of its dystrophy-preventing ability when tested on nursling rats from vitamin E-deficient mothers.[3] However, when such treated oil or vitamin E concentrate was tested on dystrophic rabbits,[4] it was ineffective in curing dystrophy. These contradictory results may be explained by variable quantitative requirements, as between functions and species, or by the uncertain results of ferric chloride oxidation; this may be incomplete even when pure tocopherol is used.[2]

To answer the question more definitely as to the biological activity of α-tocopherylquinone in preventing or curing muscle dystrophy in rabbits, dl-α-tocopherol[5] was quantitatively oxidized by gold chloride.[6] Control animals on a synthetic dystrophy-producing diet containing 5 per cent of bakers' yeast[7] succumbed in about 3 weeks. Both as preventive and cure the quinone given by mouth and parenterally to twelve young rabbits (400 to 800 gm.) in doses averaging 2 to 10 mg. per day was entirely ineffective.

[1] Wright, M. D., and Drummond, J. C., *Biochem. J.*, **34**, 32 (1940).

[2] Karrer, P., and Geiger, A., *Helv. chim. acta*, **23**, 455 (1940). Golumbic, C., and Mattill, H. A., *J. Biol. Chem.*, **134**, 535 (1940).

[3] Goettsch, M., and Ritzmann, J., *J. Nutrition*, **17**, 371 (1939).

[4] MacKenzie, C. G., and McCollum, E. V., *J. Nutrition*, **19**, 345 (1940).

[5] From ephynal generously supplied by Hoffmann-La Roche, Inc.

[6] Karrer, P., Escher, R., Fritzsche, H., Keller, H., Ringier, B. H., and Salomon, H., *Helv. chim. acta*, **21**, 939 (1938).

[7] Courtesy of Northwestern Yeast Company.

A water-soluble factor present in wheat germ has been considered an accessory agent[8] but its nature and that of the dystrophy produced by lack of it have never been indicated. Before this can be done a more complete knowledge of the requirements of the rabbit for the various B vitamins is needed. In order to be assured that the ineffectiveness of α-tocopherylquinone was not due to lack of water-soluble factors, the basal diet was in some instances enriched by an increase in the yeast content (to 20 per cent) or by the inclusion of 10 per cent of fat-free liver powder;[9] in addition massive doses of the various components of the vitamin B complex, including thiamine chloride, riboflavin, nicotinic acid, B_6, and ryzamin-B were administered separately and together. These measures had no effect on the experimental or the control animals and histological examination[10] showed that the nature of the degeneration was not altered.

Timely administration of small doses of α-tocopherol acetate to animals on either diet was preventive or curative. In one instance a single dose of 50 mg. to a 968 gm. rabbit, 16 days on the basal diet and beginning to lose weight, caused a resumption and continuance of growth for 50 days, to a weight of 1595 gm., when permanent decline in weight again began.

In the young rabbit α-tocopherylquinone can therefore not replace α-tocopherol in preventing dystrophy. Since the oxidation of tocopherol by ferric chloride may often be incomplete, the use of ferric chloride-treated diets can provide no final information regarding the dispensability of vitamin E.

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  State University of Iowa   H. A. Mattill
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[8] Morgulis, S., Wilder, V. M., and Eppstein, S. H., *J. Nutrition*, 16, 219 (1938).

[9] Courtesy of The Wilson Laboratories.

[10] We are greatly indebted to Dr. K. M. Brinkhous and the Department of Pathology.

A NEW BIOS FACTOR IN LIVER EXTRACT

Sirs:

The known bios factors to date are inositol, thiamine, β-alanine, pantothenic acid, vitamin B_6, biotin, and biotic acid. That there may be other unknown yeast growth-stimulating substances is suggested in a recent report by Williams *et al.*[1]

By fractionating liver extract we have been able to obtain a fraction which, when added to Williams' yeast growth medium, strongly stimulated the growth of a certain strain of yeast.[2] The detailed description of the preparation of this fraction which contained 1 mg. of N per cc. will be reported subsequently. It is based on the further fractionating of the phenol eluate prepared as described by Subbarow and Jacobson[3] in their isolation of the antipernicious anemia fraction of liver extract. The phenol eluate was subjected to acetone-ether precipitation, the filtrate extracted with alcohol, and the alcoholic solution, freed of alcohol, was precipitated with acetone. Most of the activity was found in the acetone extract.

We believe that this fraction contains a substance or substances distinct from the hitherto described bios factors. Inositol, thiamine, β-alanine, and vitamin B_6 separately or combined were inactive on the growth of our strain of yeast; pantothenic acid[4] had only slight effect. In the presence of 0.04 γ of thiamine, 0.04 γ of vitamin B_6, 0.005 mg. of inositol, and 1.0 γ of β-alanine per cc. of medium, the amount of yeast growth after 18 hours was identical with that in the presence of medium alone. Pantothenic

[1] Williams, R. J., Eakin, R. E., and Snell, E. E., *J. Am. Chem. Soc.*, 62, 1204 (1940).

[2] Generously supplied by Haffenreffer Brewing Company, Strain 1101.

[3] To be published. The charcoal eluate described by Subbarow, Jacobson, and Fiske (*New England J. Med.*, 214, 194 (1936)) was precipitated by ammonium reineckate and the precipitate was extracted with hot water. Reinecke acid was removed from the hot water-insoluble fraction, which was then adsorbed on charcoal and eluted with phenol.

[4] Generously supplied to us by Dr. R. J. Williams.

acid in amounts up to 1.0 γ per cc. of medium produced only a 20 per cent increase in growth.

In addition, we have been able to obtain evidence that our material differs from biotin. This evidence is as follows: The probable identity of biotin with vitamin H has been reported by György *et al.*[5] Our present preparation was assayed by Dr. György for its vitamin H activity and was found to contain 1 vitamin H unit per mg. of N. A potent vitamin H preparation (containing 1000 units per cc.) obtained from Dr. György was tested on our yeast and when used in amounts varying from 0.01 to 100 units of vitamin H gave much less growth (300 to 630 per cent increase in growth above that on the medium alone) than 0.01 vitamin H unit of our material alone (2440 per cent increase in growth). When 0.01 vitamin H unit of our material was added to 10 units of Dr. György's preparation, growth was greatly enhanced; *i.e.*, growth was more than 4 times that obtained with 100 units of Dr. György's material alone. We believe therefore that the active principle in our material differs from vitamin H and biotin.

Our active material is heat-stable, stable to acid hydrolysis, fairly stable to alkaline hydrolysis, soluble in organic solvents such as ether, alcohol, and benzene, precipitated by phosphotungstic acid, adsorbed by charcoal, and not destroyed by HNO_2 at room temperature.

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[5] György, P., Melville, D. B., Burk, D., and du Vigneaud, V., *Science,* **91,** 243 (1940).

AMINO ACID ANALOGUES OF PANTOTHENIC ACID

Sirs:

We recently reported on the biological activity of the condensation product of β-alanine and β,β-dimethyl-α,γ-dihydroxybutyric acid.[1] The comple.e chemical nature of this compound, designated pantothenic acid by Williams,[2] was reported by Williams and Major[3] and has received additional confirmation from the work of Babcock and Jukes.[4] We have extended the study of this vitamin and have tested biologically[5] several compounds in which the β-alanine component was replaced by several closely related amino acids.

The amino acid esters listed in the table were prepared and condensed with β,β-dimethyl-α,γ-dihydroxybutyric acid.[6] The method of coupling was one developed in the laboratory of Williams at Oregon State College for the linking of β-alanine with hydroxy acids and for the "partial synthesis" of pantothenic acid.[7,8]

The condensation products were tested by the microbiological procedure[5] at levels up to 6.0 γ per cc. of medium. In no case was activity observed. Synthetic pantothenic acid, prepared by the same method, approached maximum activity at a level of 0.03 γ of product per cc. of medium.

Our study indicates that substances as closely related to β-alanine as its α-amino isomer (α-alanine), its methyl homologue

[1] Weinstock, H. H., Jr., Arnold, A., May, E. L., and Price, D., *Science*, 91, 411 (1940).

[2] Williams, R. J., Lyman, C. M., Goodyear, G. G., Truesdail, J. H., and Holaday, D., *J. Am. Chem. Soc.*, 55, 2912 (1933).

[3] Williams, R. J., and Major, R. T., *Science*, 91, 246 (1940).

[4] Babcock, S. H., Jr., and Jukes, T. H., *J. Am. Chem. Soc.*, 62, 1628 (1940).

[5] The microbiological procedure for the estimation of pantothenic acid was furnished us through the courtesy of Professor R. J. Williams.

[6] Kohn, M., and Neustadter, V., *Monatsh. Chem.*, 39, 293 (1918).

[7] Weinstock, H. H., Jr., Mitchell, H. K., Pratt, E. F., and Williams, R. J., *J. Am. Chem. Soc.*, 61, 1421 (1939).

[8] Williams, R. J., and coworkers, unpublished work.

(β-aminobutyric acid), and carboxy-β-alanine (aspartic acid) are inactive when coupled with the non-nitrogenous component of pantothenic acid.[9]

The diamino acid, lysine, also failed to give an active condensation product.

Coupling of β-alanine with various 4-, 5-, and 6-carbon dihydroxy acids differing only slightly in structure from that contained in pantothenic acid has been shown to yield products of low or negative biological activity.[8,10] The inactivity of the substances obtained by alteration of the nitrogenous portion of the molecule, therefore, bears out the high structural specificity already observed for this vitamin.

Amino acid ester*	B.p. at 10 mm.	Derivative	M.p.
	°C.		°C.
l-Diethyl aspartate...............	123–125		
dl-α-Alanine ethyl ester...........		Picrate	171
dl-Lysine methyl ester............		Hydrochloride	219
β-Aminobutyric acid ethyl ester....		Picrate†	148.5–149

* An attempted preparation of the methyl ester of asparagine by treatment with diazomethane yielded a small amount of a substance which was distilled under a vacuum from a microdistillation bulb (3 mm. of Hg, bath temperature 100°). The hydrochloride melted at 183°. Calculated for $C_7H_{15}O_3N_2Cl$, C 39.90, N 7.18; found, C 39.50, 39.39, H 7.24, 7.16.

† Calculated for $C_{12}H_{16}O_9N_4$, C 40.01, N 4.48; found, C 40.17, N 4.83.

The analysis corresponds to the betaine of aspargine. Kuhn and Brydowna found that betaines could be prepared on treatment of α-amino acids with diazomethane.[11]

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[9] Since the completion of this work we have learned (private communication) that Dr. D. W. Woolley tested the α-alanine analogue with similar results.

[10] Woolley, D. W., and Hutchings, B. L., *J. Bact.*, **38**, 285 (1939); **39**, 287 (1940). Subbarow, Y., and Rane, L., *J. Am. Chem. Soc.*, **61**, 1616 (1939).

[11] Kuhn, R., and Brydowna, W., *Ber. chem. Ges.*, **70 B**, 1333 (1937).

THE PREPARATION OF COENZYME I FROM YEAST

Sirs:

The available methods for the isolation of coenzyme I from yeast[1] are tediously involved. The following are the details of a simple method which can be carried out in 1 working day.

3.2 kilos of pressed bakers' yeast are crumbled and added slowly to 2.8 liters of water at 92° with constant stirring. The suspension is filtered hot on Buchner funnels through a pad of kieselguhr. To the clear filtrate add 25 per cent basic lead acetate solution in the proportion of about 10 cc. per 100 cc. of filtrate. Small scale tests should be made of the ease of filtration. If slow, add 1 to 2 cc. more of the reagent. The filtration with suction through kieselguhr should be rapid and the filtrate clear.

Adjust the pH of the filtrate to 6.5 with 10 per cent acetic acid (just yellow to brom-thymol blue). Add 35 cc. of 25 per cent silver nitrate solution and allow the precipitate to settle. Decant the supernatant fluid, centrifuge off, and wash the precipitate thrice with water. Suspend the precipitate in 50 cc. of water and decompose with hydrogen sulfide. Filter off the silver sulfide and remove hydrogen sulfide by aeration. Pour the solution into 5 volumes of acetone and allow to stand in the cold for about 2 hours. Filter on a sintered glass funnel and wash well with acetone. Dry *in vacuo* over sulfuric acid. Yield 0.5 gm.

Estimation of total phosphorus shows 6.56 per cent instead of the theoretical 9.53 per cent. Assuming that no other phosphorus-containing substance is present, it follows that the preparation is 68 per cent pure with respect to coenzyme I. The purity was also checked by estimation of nicotinic acid and by spectrophotometric determination of the height of the 340 mμ band for dihydrocoenzyme I after reduction by the lactic enzyme system. The respective values for purity were 63 and 65 per cent. The

[1] Warburg, O., and Christian, W., *Biochem. Z.*, **287**, 303 (1936). Green, D. E., and Brosteaux, J., *Biochem. J.*, **30**, 1489 (1936). von Euler, H., Albers, H., and Schlenk, F., *Z. physiol. Chem.*, **240**, 113 (1936).

main impurity present, therefore, cannot be of a nucleotide nature. Also the fact that the spectrophotometric estimate is in agreement with the phosphorus figure rules out the presence of coenzyme II in any significant amount.

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SEPARATION OF COMPLEMENT FROM FRESH GUINEA PIG SERUM*

Sirs:

Although numerous attempts have been made to separate complement from active serum, the results have been inconclusive. In general, the experiments were designed to split off the various component parts of complement.[1] In the procedures employed in the past, few or no attempts were made to avoid denaturation of the serum proteins.   Furthermore, those who have claimed that complement resides in different fractions of serums did not give the exact method of precipitation and failed to characterize the serum fraction or fractions in question.   This may account for the belief that the serum albumins are intimately associated with complement function.

During the course of an extensive study of complement, it was discovered that complement of great activity can be separated from native serum under strictly controlled conditions.   The conditions under which separation is obtained follow.

The reagents and serums are cooled to 0°, and the preparation is carried out in a constant temperature, cold room whose temperature is maintained at 0–2°.   The ammonium sulfate solutions are filtered before use, and should have a pH of 5.8.   The pH of the 0.9 per cent NaCl solution should be maintained at 7 by the addition of K_2HPO_4; under the experimental conditions in this laboratory 1 liter of NaCl solution required 0.6 cc. of 1 M K_2HPO_4.

1 volume of fresh guinea pig serum is rapidly added with stirring to 14 volumes of a stock solution of ammonium sulfate, 2.38 or 2.40 M.   The mixture is allowed to stand for 45 minutes, after

[1] Bronfenbrenner, J., and Noguchi, H., *J. Exp. Med.*, **15**, 598 (1912). Browning, C. H., and Mackie, T. J., Immunochemical studies, Philadelphia, 180 (1925).   Parsons, E. I., *J. Immunol.*, **12**, 47 (1926).   Tokunaga, H., *Zentr. Bakt.*, **115**, 197 (1930).   Doladilhe, M., *Ann. Inst. Pasteur*, **59**, 624 (1937).

which it is centrifuged in an angle centrifuge at 2750 R.P.M. for 90 minutes. The supernatant is decanted and discarded, and the precipitate is drained against filter paper for 5 minutes. 1 volume of buffered 0.9 per cent NaCl solution is then added to the precipitate, and the mixture is vigorously stirred until the precipitate dissolves. 14 volumes of the stock ammonium sulfate solution are rapidly added with stirring, and the mixture is allowed to stand for 45 minutes. The material is now centrifuged in the angle centrifuge for 90 minutes at 2750 R.P.M., the supernatant discarded, and the precipitate drained as before. The precipitate is dissolved in 1 volume of buffered salt solution, and transferred to a dialysis bag (cellophane tube of 23 mm. width). The centrifuge bottles or tubes are rinsed with 1 volume of the buffered salt solution and the washings are also transferred to the cellophane bag. The solution is dialyzed at 0° against running, buffered salt solution for 60 hours, at the end of which time it is usually free from sulfate ions. The final product is an opalescent solution. Complement purified in this manner may show as high as 90 per cent of the original serum activity, and is inactivated by heating at 50° for 30 minutes.

Characterization of this purified complement by use of a fractional precipitation curve and by other means revealed that it is a constituent of the serum globulins. The relationship of the functional qualities of the purified complement to natural and acquired immunity is now open for study. The details of physical, chemical, and immunological characteristics will be published elsewhere.

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THE CONVERSION OF TESTOSTERONE INTO
ETIOALLOCHOLANOL-3(β)-17-ONE*

Sirs:

After the administration of testosterone to men with deficient testicular secretion, it has been possible to isolate from the urine androsterone (I) and an isomer of this androgen, etiocholanol-3-(α)-17-one (II).[1] When these experiments were repeated in the

I	II	III
Androsterone	Etiocholanol-3(α)	Etioallocholanol-3(β)
Etioallocholanol-3(α)	-17-one	-17-one
-17-one		
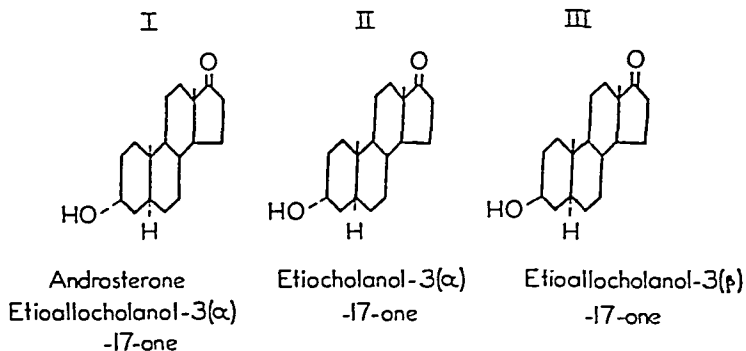
adult male guinea pig with a view to establishing these same metabolic relationships in a laboratory animal, a second isomer of androsterone was isolated which has been identified as etioallocholanol-3(β)-17-one (III). This steroid has been isolated previously from the urine of patients with adrenal cortical pathology.[2]

[1] Callow, N. H., *Biochem. J.*, **33**, 559 (1939). Dorfman, R. I., Cook, J. W., and Hamilton, J. B., *J. Biol. Chem.*, **130**, 285 (1939). Dorfman, R. I., unpublished results.

[2] Butler, G. C., and Marrian, G. F., *J. Biol. Chem.*, **124**, 237 (1938).

40 mg. of testosterone propionate dissolved in olive oil were injected subcutaneously daily for 4 days into each of four adult male guinea pigs. The pooled urine collected for a period of 7 days was thoroughly extracted with benzene after a preliminary acid hydrolysis. The ketonic neutral compounds were subjected to chromatographic adsorption on an aluminum oxide column and with carbon tetrachloride as the solvent. After adsorption on the column of the compounds dissolved in carbon tetrachloride, selective elution was carried out with mixtures of carbon tetrachloride and ethanol. From the fraction containing 0.2 per cent ethanol in carbon tetrachloride, 15 mg. of a crystalline material, m.p. 162–166°, were isolated. Recrystallization from methanol yielded a substance which melted at 169–170°. The melting point was not depressed when the compound was mixed with an authentic sample of etioallocholanol-3(β)-17-one. Both the benzoate and acetate were prepared and melted at 212–214° and 114–116°, respectively. Authentic samples of the benzoate and acetate of etioallocholanol-3(β)-17-one did not depress the melting points of the derivatives of the compound isolated.

In a second experiment, 50 mg. of testosterone propionate dissolved in olive oil were injected subcutaneously daily for 4 days into each of four adult male guinea pigs. From the urine collected from these animals over a period of 6 days, etioallocholanol-3(β)-17-one was isolated by means of its insoluble digitonide and identified. In this experiment, 16 mg. of the compound were isolated, melting at 170–171°. Attempts are being made to isolate androsterone and etiocholanol-3(α)-17-one from these urine concentrates.

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THE CONVERSION OF PROTHROMBIN TO THROMBIN, FOLLOWED BY MEANS OF THE RADIOACTIVE PHOSPHORUS ISOTOPE*

Sirs:

The use of the radioactive isotope P_{15}^{32} appears to offer a new approach to the problem of the conversion of prothrombin to thrombin under the influence of the thromboplastic lipoprotein occurring in animal tissues. It has long been a matter of conjecture whether thrombin represented a compound between prothrombin, calcium, and the thromboplastic factor (or the cephalin contained in it), or whether these substances interacted in some different manner.

The radioactive thromboplastin was isolated from rats to which radioactive Na_2HPO_4 had been administered, by a method recently elaborated for the isolation of this protein from lungs.[1] The methods for the measurement of radioactivity have been published previously.[2]

Three experiments are reported here. Prothrombin (from beef plasma)[3] was converted to thrombin by mixing a neutral solution of 260 mg. of prothrombin in 15 cc. of saline to which 10 cc. of a 0.025 M $CaCl_2$ solution had been added with a suspension of 100 mg. of the radioactive thromboplastin in 10 cc. of saline. The radioactivity of the solution of thrombin obtained by centrifugation was compared with that of control solutions resulting from the treatment of (1) a solution of crystalline albumin from horse serum, and (2) physiological saline with the radioactive thromboplastin under comparable conditions. The *thromboplastin* used had an activity of 235,000 KF units per mg. of *P. The *thrombin solution* showed a radioactivity of 332 KF units per

[1] Cohen, S. S., and Chargaff, E., unpublished data.
[2] Chargaff, E., *J. Biol. Chem.*, **128**, 579 (1939).
[3] Mellanby, J., *Proc. Roy. Soc. London, Series B*, **107**, 271 (1930).

cc. (corresponding to 614 γ of thromboplastin, or 1.41 γ of *P). The activities of the two *control solutions* were 245 and 244 KF units per cc. respectively (corresponding to 452 γ of thromboplastin, or 1.04 γ of *P). The amount of radioactivity found in the control solutions was in good agreement with measurements of the solubility of the thromboplastic protein in water. These findings showed that 1 mg. of prothrombin had taken up only 0.19 γ or 0.02 per cent of *P.

5 cc. of chicken plasma were coagulated by the addition of 40 mg. of the radioactive thromboplastin in 5 cc. of saline and of 5 cc. of a 0.025 M $CaCl_2$ solution. The *serum* had a radioactivity of 485 KF units per cc. (corresponding to 895 γ of thromboplastin or 2.06 γ of *P). The *control solutions* showed 236 KF units per cc. (corresponding to 435 γ of thromboplastin or 1.0 γ of *P).

10 cc. of a prothrombin solution (from 20 cc. of sheep plasma)[4] were mixed with 40 mg. of the thromboplastin in 5 cc. of saline and 1 cc. of a 0.1 M $CaCl_2$ solution. The *thrombin solution* after centrifugation had a radioactivity of 589 KF units per cc. (corresponding to 1085 γ of thromboplastin or 2.5 γ of *P). The *control solutions* showed 240 KF units per cc. (corresponding to 443 γ of thromboplastin or 1.04 γ of *P).

The experiments, which are being continued, show that the amount of P-containing compounds transferred to the thrombin, if at all significant, is extremely small and could not have been detected by other methods.

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[4] Eagle, H., *J. Gen. Physiol.*, **18**, 531 (1935).

A NOTE ON AN ASSAY METHOD FOR PANTOTHENIC ACID IN HUMAN BLOOD*

Sirs:

Pennington, Snell, and Williams[1] have devised a bacteriological method for assaying crude extracts for pantothenic acid. In order to determine the pantothenic acid content of human blood, we have developed the following modification of their technique.

Fresh venous blood is citrated to prevent clotting. 1 cc. of blood is laked with 9 cc. of water and the resulting solution is added to tubes of medium in duplicate concentrations ranging from 0.1 to 0.3 cc. of original blood.[2] The tubes are plugged and autoclaved at 15 pounds pressure for 15 minutes. This procedure causes the precipitation of the blood proteins and necessitates a vigorous shaking of the tubes after inoculation. The tubes are also shaken (by hand) at the end of the first 24 hours of incubation. The acid produced is titrated at the end of 72 hours and the amount of pantothenic acid present is read from the standard curve. The presence of the precipitate causes some inaccuracy in reading the end-point, but this is reduced to a minimum if the titration is made in a strong light and the precipitate is allowed to settle so that the color of the supernatant liquid may be easily seen.

The variation between duplicate concentrations of the same blood is not more than 0.05 to 0.1 cc. of alkali. Agreement of values at different dosage levels indicates that the test is specific for pantothenic acid when the blood concentration does not go above 0.3 cc. The recoveries of added pantothenic acid by this procedure are 95 to 100 per cent. When higher blood concentra-

[1] Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **135**, 213 (1940).

[2] The medium, organism, and technique used are those reported by Pennington, Snell, and Williams (foot-note 1).

tions are used, they drop to 60 to 80 per cent as do the values on the original blood. The addition of a pantothenic acid-free blood filtrate to the basal medium did not increase the specificity or accuracy of the test. The pantothenic acid content of the blood of eighteen normal persons fell within the limits of 0.19 to 0.32 γ per cc., and averaged 0.225 γ per cc.

Using this method we have found (1) that the pantothenic acid content of the blood of eighteen normal persons varied within fairly narrow limits, (2) that samples taken from the same person at different times during the day or on successive days show less than 0.02 γ per cc. variation, (3) that autolysis of blood does not liberate additional pantothenic acid, (4) that the blood of twenty-eight patients with pellagra, beriberi, and riboflavin deficiency shows a decreased pantothenic acid content, averaging 0.05 to 0.09 γ (23 to 50 per cent) below the normal average.

These findings suggest that pantothenic acid is important in human nutrition.

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IDENTIFICATION OF THE RICE FACTOR. THE ESSENTIAL NATURE OF THE GLYCINE COMPONENT

Sirs:

Earlier reports[1] have described the division of the chick "rice factor" into two components, glycine and glucuronic acid or compounds related to each. We desire at this time to present some results of further studies on the essential nature of glycine for the chick. The basal diet and methods used were practically the same as given in earlier papers[1] and will be described in detail in a later report. Results are listed in the table. These have been confirmed by other experiments.

The most interesting feature of these results is that creatine is more effective than glycine in promoting growth. A logical interpretation would be that glycine is required for the synthesis of creatine and that in a deficiency of glycine creatine synthesis is retarded to an extent which reduces the rate of growth of the chick. Strong evidence that glycine is a precursor of creatine for other species has been presented[2] but, apparently, there has been no proved relation of these compounds to growth.

Many of the chicks on inadequately supplemented diets develop a markéd dystrophy or attenuation of the muscles and a general weakness. Creatine determinations made on leg muscle tissue have shown that the basal, sodium acetate, and glycine groups all have about the same muscle creatine content but in the case of the group fed creatine the muscle creatine content is higher. The lower muscle creatine content may represent a minimum which is maintained at the expense of muscle size.

Apparently the acetates can also be utilized, since they serve as well as glycine. On the other hand, glycolic acid (hydroxy-

[1] Almquist, H. J., Stokstad, E. L. R., Mecchi, E., and Manning, P. D. V., *J. Biol. Chem.*, 134, 213 (1940). Almquist, H. J., Mecchi, E., Stokstad, E. L. R., and Manning, P. D. V., *J. Biol. Chem.*, 134, 465 (1940).

[2] Borsook, H., and Dubnoff, J. W., *Science*, 91, 551 (1940). Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, 133, 633 (1940).

Comparison of Various Compounds As Substitutes for Glycine in Chick Diets

Supplement to basal diet	Level	Basal gain made	Creatine in leg muscle*
	per cent	*per cent*	*mg. per gm.*
None		100	3.18
Glycine	0.75	120	
"	1.50	124	3.35
Sodium acetate	1.00	129	
" "	2.00	129	3.19
Ammonium acetate	2.00	134	
Creatine	0.75	141	
"	1.50	150	4.12
Glycolic acid	1.00	87	
Betaine	1.00	106	
Guanidine	0.50	103	
β-Alanine	0.10	96	
Choline	0.25	101	

     * An average value for at least nine chicks per group.

acetic acid) has repeatedly proved ineffective or detrimental. Betaine, guanidine, β-alanine, and choline have shown little or no effect in these tests.

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THE STABILITY OF HYDROGEN-CARBON LINKAGES IN GLUTAMIC ACID

Sirs:

Preliminary to the study of the metabolism of deutero glutamic acid, we have investigated the stability of its hydrogen atoms. Those of the two carboxyl groups and of the amino group of course exchange at a very rapid rate. It has been shown that the compound also contains some stably bound hydrogen[1] and some that exchanges at a very slow rate when the glutamic acid is heated with 20 per cent HCl in heavy water. This latter reaction is half completed at 100° in about 4 days. Hydrogen of this type has been designated semilabile.[2] It is not in the α position, for introduction of deuterium does not run parallel with racemization, and the succinic acid obtained by oxidation[3] of glutamic acid so treated contains all of the deuterium originally present. The semilabile hydrogen must therefore be in either the β or the γ position, or both.

It has now been shown that the hydrogen in the β position, like that in the α position, is stable. By the catalytic hydrogenation of α-ketoglutaric acid with deuterium gas in presence of ammonia, glutamic acid is formed from which no deuterium is removed on prolonged boiling with 20 per cent HCl. According to this mode of synthesis deuterium could have been introduced only at the α and β positions, and experiment has shown that none is introduced into glutamic acid itself under the conditions of hydrogena-

[1] Foster, G. L., Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **125**, 13 (1938).

[2] Rittenberg, D., Keston, A. S., Schoenheimer, R., and Foster, G. L., *J. Biol. Chem.*, **125**, 1 (1938).

[3] It is necessary to avoid high temperature in these reactions, as the hydrogen atoms of succinic acid slowly exchange under these conditions. Boiling succinic acid in 20 per cent HCl containing 3.3 atom per cent D for 1 hour results in the introduction of 0.07 atom per cent deuterium in the succinate ion. This corresponds to a labilization of 0.1 hydrogen atom per mole.

tion with deuterium.   A sample of the synthetic glutamic acid containing 15.5 atom per cent deuterium was degraded to succinic acid with chloramine-T; this process resulted in the loss of some of the deuterium present in the starting material.   The barium succinate isolated contained 28.4 atom per cent deuterium.   If no deuterium had been present in the α position of the glutamic acid, the barium succinate should have contained 35 atom per cent D.   The difference indicates that the hydrogen attached to the α-carbon atom contained about 25 atom per cent deuterium. As the hydrogen atoms at the α and β positions are stable towards acid, the semilabile hydrogen atoms of glutamic acid must be those attached to the γ-carbon atom.

By the methods outlined it is thus possible to determine the position of deuterium in any deutero glutamic acid.   If present in the γ position, it is slowly removable by boiling with HCl in ordinary water.   If the deuterium is not removable, it must be located in the α or β position, or both.   Degradation of the deutero glutamic acid into succinic acid and isotope analysis of the latter will reveal how much was located at the β- and how much at the α-carbon atom.

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RADIOACTIVE PHOSPHORUS AS AN INDICATOR OF PHOSPHOLIPID METABOLISM

XII. FURTHER OBSERVATIONS ON THE EFFECTS OF AMINO ACIDS ON PHOSPHOLIPID ACTIVITY OF THE LIVER*

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Three amino acids have been shown to accelerate phospholipid turnover in the liver: methionine, cystine, and cysteine (1). In short intervals after the administration of radioactive phosphorus, greater amounts of labeled phospholipid are deposited in the livers of rats fed these three amino acids than in the livers of control animals. In the case of methionine, this finding lends support to the view that the rate of phospholipid turnover is a factor determining the fat content of the liver. Methionine stimulates phospholipid turnover (1) and prevents the deposition of excessive amounts of fat in the liver (2, 3). The observation, however, that cystine and cysteine stimulate phospholipid turnover appears incompatible with their lipotropic actions, for prolonged feeding of both does not prevent fatty infiltration in the liver. Although this discrepancy merits further investigation, it was nevertheless pointed out that an increased *phospholipid turnover* was observed in this laboratory after *single* feedings of cystine or cysteine, whereas *lipotropic actions* were detected only after their *prolonged* administration (4). Deleterious effects have repeatedly been observed after prolonged feeding of cystine (5-7). In this connection it would be of interest to know whether a single feeding of cystine could produce a lipotropic effect, but it seems very unlikely at present that a change in the fat content of the liver could be detected after so short an interval.

Amino acids other than methionine have been shown to possess no lipotropic action (8), although such an effect has recently been demonstrated by Singal for the betaine of cystine (9).   It seemed desirable to extend our observations on the effects of other amino acids on the rate of phospholipid turnover as measured with radioactive phosphorus. This has been done in the present investigation.   In addition, a study of related compounds was undertaken with the view of obtaining evidence for a particular grouping or linkage responsible for phospholipid stimulation.

EXPERIMENTAL

The results shown were obtained from separate analyses of 292 rats.   Average values only are recorded.   The experimental procedures were essentially the same as those previously employed (1). The rats were fed the high fat-low protein diet for 3 days and then injected with radioactive phosphorus.   The amino acids and other compounds tested here were suspended in gum tragacanth solution and fed by stomach tube at the same time that radioactive phosphorus was administered.   The control animals received the radioactive phosphorus and the pure gum solution.   The animals were sacrificed 7 to 8 hours after they received the radioactive phosphorus and the livers were excised.   The liver phospholipids were extracted and their radioactivity determined in the manner previously described (1, 10).   Animals were carefully paired, so that amino acid-fed rats were compared with control rats of the same weight and sex.

Results

The effects of seven amino acids on phospholipid turnover of the liver are shown in Table I.   Each amino acid was fed to groups of eight or twelve rats and the content of radioactive phospholipid in their livers compared with that found in an equal number of control animals that were observed simultaneously. Two amino acids, methionine and cysteine, which have already been shown to have a stimulating effect upon phospholipid activity of the liver, served as a further basis for comparison.   In the present study, even as small an amount as 35 mg. of methionine (Table I) is shown to have a stimulating effect on the rate of phospholipid formation in the liver.   7 hours after the adminis-

tration of radioactive phosphorus, the average amount of labeled phospholipid deposited in the liver of the rats that had received methionine was 22 per cent greater than that of the control animals. Cysteine was fed in a 200 mg. dose (Table I), and the average value found for the phospholipid activities of eight rats

TABLE I

*Effect of Amino Acids on Phospholipid Activity of Liver**

Amino acid†	Amount fed	No. of rats	Average activity of fed rats	Average activity of control rats	Per cent change in fed rats
	mg.				
Cysteine	200	16	3.32 ± 0.20‡	2.61 ± 0.17‡	27
Methionine	35	24	3.90 ± 0.14	3.19 ± 0.13	22
Glycine	100	16	2.66 ± 0.18	2.70 ± 0.12	−1
	200	16	2.87 ± 0.19	2.86 ± 0.14	0
l-Alanine	200	16	3.31 ± 0.25	3.16 ± 0.11	5
l-Tyrosine	200	16	3.09 ± 0.25	3.16 ± 0.11	−2
	400	16	3.26 ± 0.04	3.34 ± 0.04	2
d-Glutamic acid	200	16	2.68 ± 0.13	2.86 ± 0.14	−6
	200	24	3.06 ± 0.17	2.97 ± 0.10	3
	400	24	3.01 ± 0.07	2.97 ± 0.10	1
l-Asparagine	200	16	3.34 ± 0.14	3.09 ± 0.13	8
	200	16	2.62 ± 0.09	2.61 ± 0.17	0
l-Proline	200	16	3.34 ± 0.15	3.09 ± 0.13	8
	200	16	2.33 ± 0.12	2.61 ± 0.17	−10
dl-Serine	200	16	3.50 ± 0.27	3.37 ± 0.13	4

* The percentage of the administered labeled phosphorus found as phospholipid in the whole liver. The livers were excised 7 to 8 hours after the injection of radioactive phosphorus.

† All compounds fed except proline and tyrosine were Pfanstiehl, c.p. grade. Proline, c.p., was obtained from the Amino Acid Manufactures, University of California at Los Angeles. The tyrosine used was an Eastman Kodak product.

‡ Standard error of the mean.

was 27 per cent greater than the average control value. This compares favorably with previous findings, in which the average values of the treated animals showed increases of 39, 17, 40, and 21 per cent in four separate experiments.

Glycine was fed in two amounts, 100 and 200 mg. (Table I). An average of 2.66 per cent of the labeled phosphorus was found

as phospholipid in the whole livers of the group of rats that had received 100 mg. of glycine, whereas the livers of the animals that had received 200 mg. of glycine contained 2.87 per cent as phospholipid. The respective average control values were 2.70 and 2.86 per cent.

l-Alanine also failed to change the rate at which labeled phosphorus is deposited as phospholipid in the liver (Table I). The livers of eight rats that received 200 mg. of *l*-alanine showed an

TABLE II

*Effect of Compounds Other Than Amino Acids on Phospholipid Activity of Liver**

200 mg. were fed to sixteen rats in each case.

Compound† fed	Average activity of fed rats	Average activity of control rats	Per cent change in fed rats
Taurine	3.06 ± 0.11‡	2.76 ± 0.11‡	10
Di-(β-hydroxyethyl) sulfoxide	3.10 ± 0.21	2.76 ± 0.11	12
Creatine	2.53 ± 0.08	2.37 ± 0.17	7
	2.78 ± 0.12	2.64 ± 0.08	5
	2.76 ± 0.13	2.58 ± 0.27	7
Sarcosine	3.59 ± 0.15	3.37 ± 0.13	6

* The percentage of the administered labeled phosphorus found as phospholipid in the whole liver. The livers were excised 7.5 to 8 hours after the injection of radioactive phosphorus.

† The creatine and sarcosine used were Pfanstiehl, c.p., products. Taurine and di-(β-hydroxyethyl) sulfoxide were synthetic preparations.

‡ Standard error of the mean.

average activity of 3.31 per cent, whereas that found in the livers of the control group was 3.16 per cent.

Five other amino acids, namely *l-tyrosine, d-glutamic acid, l-asparagine, l-proline,* and *dl-serine,* failed to influence the rate of phospholipid turnover in the liver as measured with radioactive phosphorus (Table I). In some cases amounts as high as 400 mg. were fed by stomach tube at a single time, but even these failed to stimulate the liver to increased phospholipid activity.

The special position of the sulfur-containing amino acids suggested the use of taurine and of di-(β-hydroxyethyl) sulfoxide.[1]

[1] The use of this compound was suggested by Dr. H. Tarver.

The former is derived from amino acids, and its sulfur is in a higher state of oxidation than that found in the amino acids cysteine, cystine, and methionine. Di-(β-hydroxyethyl) sulfoxide is a sulfur-containing compound naturally occurring in the adrenal gland. It was isolated by Reichstein and Goldschmidt (11) from the lipid fraction of the adrenal gland in which it is believed to be combined with fatty acids through an ester linkage. In the present study the effects of taurine and of di-(β-hydroxyethyl) sulfoxide upon the rate of phospholipid turnover in the liver of the rat was determined (Table II). The feeding of 200 mg. of each failed to influence phospholipid activity in the liver.

Two other compounds were tested in a similar manner: creatine and sarcosine. Platt (12) has shown that creatine is lipotropically inactive. The influence of creatine on phospholipid activity of the liver is shown in Table II. Animals were fed 200 mg. of creatine and sarcosine, and the labeled phospholipid content of their livers was determined 7.5 hours later. No change in the phospholipid activity of the liver was observed. The average values observed in the fed rats did not differ by more than 7 per cent from control values.

<div align="center">DISCUSSION</div>

The uniformly negative results obtained with glycine, alanine, tyrosine, glutamic acid, asparagine, proline, and serine show that the ability to stimulate phospholipid turnover in the liver is not common to all amino acids; indeed, it appears at present that only the three sulfur-containing amino acids, methionine, cystine, and cysteine, possess this property. The ineffectiveness of glycine, alanine, serine, asparagine, and glutamic acid indicates that the unique structural grouping responsible for phospholipid stimulation is not the α-amino acid of a particular chain length. The two sulfur-containing compounds, taurine, which is derived from cystine, and di-(β-hydroxyethyl) sulfoxide, a compound found in the adrenal gland, were also inactive. It may be of significance here that both of these compounds contain sulfur in a higher state of oxidation than that in the three active amino acids.

The radioactive phosphorus used in this investigation was supplied by members of the Radiation Laboratory under the direction of Professor E. O. Lawrence, to whom our thanks are due.

SUMMARY

1. Amino acids differ in their capacity to stimulate phospholipid activity of the liver as measured with radioactive phosphorus. The stimulating effects of methionine and cysteine have been confirmed. The following amino acids have been shown to be inactive in this respect: glycine, alanine, serine, tyrosine, proline, glutamic acid, and asparagine.

2. Taurine, creatine, di-(β-hydroxyethyl) sulfoxide, and sarcosine failed to stimulate phospholipid turnover in the liver.

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ON THE MECHANISM OF COCARBOXYLASE ACTION; A REINVESTIGATION*

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In a preceding publication from this laboratory (1) evidence was presented which suggested that cocarboxylase, in the course of its physiological function, undergoes a cyclic change involving reversible oxidation-reduction rather than the formation of a catalytically active substituted imino acid, as postulated by Langenbeck. These conclusions were based on the failure of thiamine to exhibit certain properties typical of primary amines, in spite of the presence of a NH_2 group in the pyrimidine ring, and on the apparent activity of thiamine pyrophosphate (cocarboxylase) after reduction in vitro both in the avitaminotic pigeon and in the enzymatic yeast test system. Subsequently, however, Barron and Lyman[1] found that partially reduced cocarboxylase, when tested on Na_2HPO_4-washed yeast at concentrations considerably lower than those employed by the present authors, shows an activity approximately proportional to the amount of residual non-reduced coenzyme present in the preparation. Accordingly we have reinvestigated the question of the activity of dihydrococarboxylase.

EXPERIMENTAL

The technique used in the cocarboxylase activity determinations and in the reduction of cocarboxylase by catalytically activated

[1] Unpublished experiments, kindly communicated to the authors by Dr. E. S. Guzman Barron.

hydrogen under manometric control was the same as that described previously (1). However, the evaluation of the activity of the reduced coenzyme preparations was rendered more critical in two respects.

Previously the activity of reduced coenzyme preparations was determined by computing the ratio of the CO_2 produced from pyruvic acid by the reduced material to the CO_2 produced by the same amount of cocarboxylase. This method of assay, however, is liable to yield erroneous results when large amounts of coenzyme are employed. This is due to the saturation of the active protein with coenzyme in the higher concentration ranges; additional coenzyme produces only little additional activity under such conditions. It was found advisable, therefore, to ascertain the activity of coenzyme samples by determining the amount of reduction product which will produce the same volume of CO_2 in the carboxylase test system as a fixed amount of non-reduced cocarboxylase.

In the earlier work the degree of reduction had been estimated from the decrease in hydrogen gas pressure in the manometers during the reduction. This procedure was found to be unreliable, since samples of coenzyme, which according to the manometric readings had taken up 1 mole of hydrogen per mole of coenzyme, upon chemical analysis were found still to contain a certain fraction of non-reduced cocarboxylase. In the present experiments the actual degree of reduction was determined by analyzing the reaction product for non-reduced thiamine pyrophosphate by the colorimetric method (2) of Melnick and Field.[2]

Eight reduction experiments were performed, with colloidal palladium as the hydrogenation catalyst. In four experiments the reaction was allowed to proceed until the manometer readings indicated that approximately 1 mole of hydrogen had been absorbed per mole of cocarboxylase. The coenzyme used was a synthetic preparation kindly placed at our disposal by Merck and Company, Inc. In four experiments the reduction was interrupted by the admission of air after approximately 0.5 mole

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[2] We wish to acknowledge with thanks the kind cooperation of Dr. Daniel Melnick of the Department of Internal Medicine, University of Michigan, in performing the chemical determinations of cocarboxylase reported in this paper.

of hydrogen had been taken up. The reduction products thus obtained were assayed for cocarboxylase activity by the manometric procedure described previously (1) at various levels of coenzyme concentration. The amount of non-reduced cocarboxylase in six of the preparations was determined by the colorimetric method. The results are compiled in Table I, an inspection of which shows that there exists satisfactory agreement between the cocarboxylase activity and the amount of residual non-reduced coenzyme in these preparations.

TABLE I

Cocarboxylase Activity and Content in Non-Reduced Coenzyme of Cocarboxylase Preparations after Treatment with Palladium-Hydrogen

Preparation	Amount of hydrogen absorbed per mole coenzyme	Residue of non-reduced cocarboxylase (chemical determination)	Amount of reduction product used in yeast test system	Cocarboxylase activity as determined with calibration curve
	moles	*per cent*	γ	*per cent*
A	1.0	19	2	32
			5	29
			15	25
B	1.0		2	30
			5	36
			20	36
C	1.0		10	13
D	1.0	22	0.8	25
			1.6	25
E	0.41	43	1.2	47
F	0.50	33	1.2	29
G	0.53	29	1.2	31
H	0.44	47	1.2	50

In the light of these findings some of the observations reported in the preceding paper (1) are open to reinterpretation. The activity of several preparations which had been allowed to absorb 1 mole of hydrogen per mole of coenzyme was found at that time to be of the same order as that of the non-reduced coenzyme. Those findings may be explained by assuming (1) that a certain fraction of the coenzyme had escaped reduction and (2) that the use of relatively large amounts of the reduction product (*e.g.* 12 to 25 micrograms) in the yeast test led to an exaggerated value

for its activity.   The complete lack of activity of a preparation which had absorbed 2.33 moles of hydrogen has been ascribed to irreversible "overreduction."   The possibility must now be considered that the loss of activity was at least partly due to the hydrogenation of all of the coenzyme present in the sample. Finally it was reported in that paper that 15 micrograms of reduced cocarboxylase were able to relieve the polyneuritic symptoms in avitaminotic pigeons.   This finding may also be ex-

TABLE II

Biological Activity of Reduced* and Non-Reduced Thiamine Pyrophosphate in Two Different Test Systems

Amount of coenzyme added	*Bacillus delbrückii†*		*Yeast‡*	
	Non-reduced cocarboxylase	Reduced preparation	Non-reduced cocarboxylase	Reduced preparation
	O_2 absorbed in 30 min.		CO_2 liberated in 30 min.	
γ	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*
0.2	18	4		
0.4	33	7	86	
0.8	56	12	148	44
1.2	72	18		
1.6				82

* The reduced preparation contained 22 per cent non-reduced cocarboxylase, as determined colorimetrically (Melnick and Field method).

† 0.8 cc. of washed bacteria suspension (approximately 80 mg.) in phosphate buffer at pH 6.5, 0.05 cc. of 0.1 M $MgCl_2$, 1 microgram of flavin-adenine dinucleotide, and 0.05 cc. of 1 M sodium pyruvate were present in each manometric vessel; 0.1 cc. of 5 N NaOH in the center well; 37.5°.

‡ 100 mg. of washed yeast, 5 mg. of pyruvic acid, and 0.1 mg. of Mg (as $MgCl_2$) were present in each vessel; pH 6.1; 37.5°.

plained by assuming that this preparation contained about 20 per cent of unchanged coenzyme.

It might be argued that, since the decarboxylation of pyruvic acid by yeast does not involve an oxidation of the substrate, it may likewise involve no oxido-reductive cycle of the coenzyme and consequently be unsuitable for demonstrating biological activity for dihydrococarboxylase.   From this point of view, *Bacillus delbrückii* should represent a more appropriate test object, inasmuch as Lipmann (3) has shown that here thiamine

pyrophosphate functions as the coenzyme in a system capable of the oxidative decarboxylation of pyruvic acid to CO_2 and acetic acid. However, the data presented in Table II indicate that here too the activity exhibited by a reduced cocarboxylase preparation may be satisfactorily accounted for by that fraction of coenzyme which has escaped reduction.[3] Dr. E. S. G. Barron, in unpublished experiments, has obtained similar, negative results in this test system.

SUMMARY

The experiments presented in this report indicate that the reduction of cocarboxylase by hydrogen, activated by palladium, yields a catalytically inactive product. However, the results do not rule out the possibility that thiamine pyrophosphate may undergo a cyclic change of oxido-reductive character during the *enzymatic* breakdown of pyruvic acid.

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A STUDY OF THE URINARY EXCRETION OF VITAMIN B₆ BY A COLORIMETRIC METHOD

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Chemical methods for the determination of biological substances, in contrast with animal assays, offer a considerable saving in time, and the analytical data are usually more precise and reproducible. For these reasons, a study of the vitamin B₆-indophenol color reaction has been in progress in these laboratories for some time (1). In the course of this work a procedure has been devised whereby solutions containing a minimum of 2 micrograms of the vitamin per cc. can be analyzed with precision (2). The general applicability of this method, the nature of interfering substances, and the specificity of the test are under investigation. Details of this work will be presented elsewhere.

Method

It has been found that this method may be used in a very simple form to measure the urinary excretion of vitamin B₆ administered orally or parenterally. This application of the method depends upon the analysis of samples of urine obtained before and after the administration of the vitamin. Both analyses are necessary because interfering substances present in the preadministrative or control sample give rise to a small concentration of colored reaction products. This concentration, measured in the Evelyn colorimeter with a No. 660 filter to reduce the influence of extraneous colors, is subtracted from the concentration found in the postadministrative sample. The difference is the vitamin B₆ output. This is based on the assumption, however, that the interfering substances are the same in both samples of urine, which seems likely under the conditions employed.

In general, the conditions used are such as might be used clinically in a saturation test of vitamin B_6 deficiency. A fasting subject was given water to flush the urinary tract. The urine was discarded. After 1 hour, the control sample was taken and the bladder was emptied. At this time additional water was
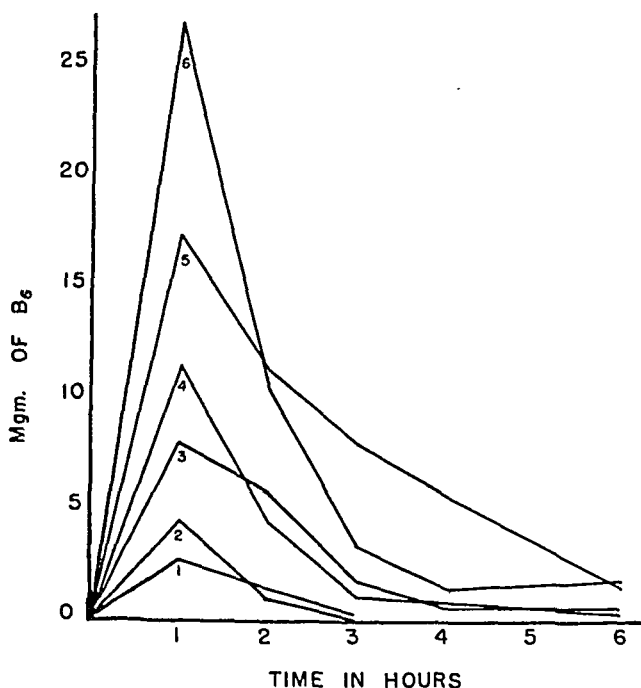
FIG. 1. Excretion of vitamin B_6 following oral administration. Curve 1, Dog 51, dose 50 mg.; Curve 2, Dog 58, dose 50 mg.; Curve 3, Dog 31, dose 100 mg., 6 hour output 16.8 per cent; Curve 4, Dog 97, dose 100 mg., 6 hour output 18.4 per cent; Curve 5, Dog 92, dose 200 mg., 6 hour output 22.0 per cent; Curve 6, Dog 31, dose 200 mg., 6 hour output 22.1 per cent, Dog 31, dose 500 mg., 6 hour output 20.3 per cent, Dog 97, dose 500 mg., 6 hour output 18.7 per cent. For Curves 1, 2, and 5 the urine samples were taken by catheter.

given to provide an adequate diuresis and the vitamin was injected intravenously or given orally. Postadministrative samples were then taken at measured intervals of time. Under these conditions, interfering substances should be essentially the same in both samples, although the concentration might be slightly lower in the postadministrative samples. This would tend to

give low, rather than high results. Urine samples taken from test subjects were analyzed both by the chemical method and by the curative assay with vitamin B₆-deficient rats (3) and the results were found to be in excellent agreement. Furthermore,
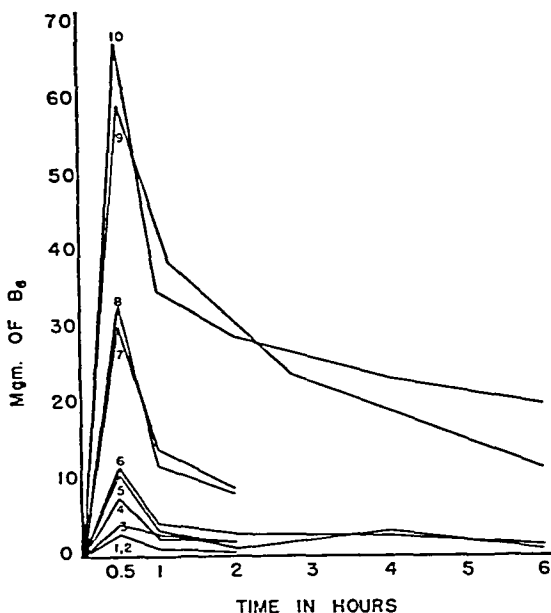
FIG. 2. Rate of excretion following the intravenous administration of vitamin B₆. Curve 1, Dog 31, dose 25 mg., 1 hour output 17.2 per cent; Curve 2, Dog 97, dose 25 mg., 1 hour output 16.8 per cent; Curve 3, Dog 97, dose 50 mg., 1 hour output 15.0 per cent; Curve 4, Dog 31, dose 50 mg., 1 hour output 20.8 per cent; Curve 5, Dog 97, dose 100 mg., 1 hour output 14.4 per cent; Curve 6, Dog 97, 100 mg., 1 hour output 16.5 per cent; Curve 7, Dog 31, dose 250 mg., 1 hour output 18.0 per cent; Curve 8, Dog 97, dose 250 mg., 1 hour output 18.0 per cent; Curve 9 (urine by catheter), Dog 66, dose 500 mg., 1 hour output 20.8 per cent; Curve 10, Dog 97, dose 500 mg., 1 hour output 20.5 per cent.

100 per cent recovery was obtained upon the addition of vitamin B₆ to samples of both dog and human urine. Nevertheless, in order to eliminate further the possibility of variations in urine samples, both the control and postadministrative samples of urine were diluted equally until the control urine gave a blank, or

very faint color reaction.   The concentration of the postadminis-trative sample was then reckoned directly from a calibratiou curve, or by difference as the case required.   This dilution reduces the sensitivity of the test, and may be a handicap if doses lower than 50 mg. are required in the development of a saturation test in man.

Experiments in Dogs—Six dogs, weighing 7 to 15 kilos and maintained on a diet of Purina Dog Chow, were used.   Control

<div align="center">TABLE I</div>

Vitamin B₆ Output in Human Subjects after Intravenous and Oral Administration

Subject No.	After intravenous administration of 50 mg. vitamin B₆					Total output 4 hrs. after oral administration of 100 mg. vitamin B₆
	15 min.	30 min.	45 min.	60 min.	Total output	
	mg.	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
1	3.3	1.0	0.2	0.0	9.0	7.6
2	3.1	0.7	0.2	0.1	8.1	8.7
3	3.9	1.2	0.2	0.4	11.4	9.4
4	3.6			2.2	11.6	7.6
5	2.4			0.9	6.6	5.1
6	2.9			0.7	7.2	7.5
7	3.7			0.3	8.0	9.4
8	4.3			0.3	9.2	7.6
9	3.1			0.5	7.2	4.5
10	5.2			0.7	11.8	8.8
11	4.5			0.3	9.6	7.2
12	3.1			0.0	6.2	
13	3.5			0.1	7.2	
Average..............................					8.7±1.6	7.6±1.1
Maximum deviation......................					3.1	3.1

samples of urine and postadministrative samples were taken either by catheterization or through a bladder fistula at the time intervals indicated in Figs. 1 and 2.   A point-line method of plotting was used for clarity in presenting these data.

Fig. 1 shows the urinary output of fasting dogs following the oral administration of the vitamin.   Maximum excretion oc-curred within 1 hour, indicating rapid absorption from the gastro-intestinal tract.   In fed dogs (recoveries shown in the legend

under Fig. 1) maximum excretion was delayed 2 to 3 hours. In all experiments 20 per cent of the vitamin (± 1, maximum deviation 3.2 per cent) was recovered within 6 hours.

After the intravenous injection of doses of the vitamin varying from 25 to 500 mg. per dog, the maximum output was attained within 30 minutes (Fig. 2). Within 1 hour, 18 per cent of the vitamin was excreted, regardless of the weight of the animal. This approximates the 6 hour output following oral administration.

Experiments in Man—A group of thirteen non-fasting, apparently healthy, adult male subjects weighing 60 to 85 kilos were given 50 mg. of the vitamin intravenously. Postadministrative samples were then taken at the intervals shown in Table I. The results obtained are more variable than those found in our study with dogs. This may be due to the non-fasting condition of the subjects.

After the oral administration of 100 mg. of the vitamin to a group of eleven non-fasting male subjects, an average recovery of 7.6 per cent of the vitamin was obtained at the end of 4 hours.

DISCUSSION

It has been reported (2) that 50 to 70 per cent of test doses of vitamin B_6 was recovered in normal rat urine regardless of the mode of administration of the vitamin. In fasting dogs, urinary excretion of the vitamin is essentially complete within 6 hours after oral administration, but there was only 20 per cent recovery. An almost equal recovery (18 per cent) was obtained in dogs within 1 hour after intravenous administration of the vitamin. These results were remarkably constant, regardless of the weights of the animals and the doses (25 to 500 mg.) administered. In non-fasting human subjects only 8.7 per cent of a 50 mg. intravenous dose of the vitamin was recovered within 1 hour, although excretion appeared to be practically complete by this time.

In earlier experiments (2) it was observed that the fraction of the vitamin excreted in the urine of deficient rats was smaller than that excreted by normal animals. However, the amounts of the vitamin given (100 micrograms per rat, or approximately 2 mg. per kilo) were too small to permit a quantitative interpretation of the results obtained. Nevertheless, it was believed that

at this same dose level (2 mg. per kilo) or even smaller doses, the urinary output of the vitamin could be measured quantitatively in larger animals. This is amply substantiated by the present work. The fraction of the vitamin excreted following doses of 2 to 3 mg. per kilo has been measured in the dog, and the output following less than 1 mg. per kilo has been quantitatively measured in human subjects.

SUMMARY

1. A colorimetric method based on the indophenol reaction has been used to study the excretion of test doses of vitamin B₆ in the normal dog and man.

2. The short interval between oral administration of the vitamin and its appearance in the urine indicates that it is rapidly absorbed from the gastrointestinal tract and readily excreted.

3. After the intravenous administration of the vitamin in doses ranging from 25 to 500 mg., 18 per cent of the vitamin was recovered from the urine of dogs within 1 hour. After oral administration, these dogs excreted 20 per cent of the vitamin within 6 hours.

4. In a group of apparently healthy human subjects, an average of 8.7 per cent of a 50 mg. intravenous dose of the vitamin was recovered within 1 hour, while 7.6 per cent of a 100 mg. oral dose was recovered within 4 hours.

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THE COLORIMETRIC ESTIMATION OF 17-KETOSTEROIDS AND THEIR APPLICATION TO URINE EXTRACTS*

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Because of the advantages of colorimetry in comparison with the biological assay, considerable interest has been shown in the colorimetric determination of androsterone. Zimmermann (1) based his method on certain theoretical work done by von Bittó (2), Reissert (3), and Weise and Tropp (4), but it was not until papers by Wu and Chou (5), Zimmermann (6), and Callow *et al.* (7) appeared that the method received wide-spread attention.

Our purpose was to evaluate the method by investigating the optimum conditions for the color reaction, its specificity, and its correlation with the capon assay for androgens (8).

Table I shows the details of various modifications of Zimmermann's colorimetric method.

Reagents—

2 per cent *m*-dinitrobenzene in 95 per cent redistilled EtOH. Eastman Kodak No. 99 *m*-dinitrobenzene was used. If in the course of the determination a precipitate is encountered caused by the *m*-dinitrobenzene, either another lot or a purification according to Callow *et al.* (7) should be tried. A concentration of 2 per cent was chosen because higher concentrations might lead to precipitation in the final dilution. The *m*-dinitrobenzene solution can be used for 10 days, after which time it gives low readings with standard androsterone solutions. Redistilled 95 per cent EtOH was used as a solvent for the *m*-dinitrobenzene and steroids because less blank color was encountered than with a variety of other kinds of ethanol.

5.00 N aqueous KOH. Eimer and Amend electrolytic KOH

TABLE I

Details of Various Modifications for Colorimetric Determination of Androsterone

Worker No.[*]	Solvent for			Reagent concentration			Reagent volume			
	Sterol	m-Dinitrobenzene	KOH	m-Dinitro-benzene (per cent)	KOH (N)	KOH (per cent)	Sterol (cc.)	m-Dinitro-benzene (cc.)	KOH (cc.)	Total (cc.)
1	Alcohol	Alcohol	Distilled H$_2$O	1	2.7	15	0.3	0.065	0.05	0.415
2	Absolute EtOH	Absolute EtOH	Absolute EtOH	2	1.8	10	0.25	0.20	0.15	0.060
3	"	"	Distilled H$_2$O	1	3.0		2 parts	1 part	1 part	4 parts
4	60% EtOH	95% EtOH	"	2	2.7	15	5.0	1.0	1.0	7.0
5	Commercial absolute EtOH	Commercial absolute EtOH	Commercial absolute EtOH	2	2.5		0.2	0.2	0.2	0.6
6	Redistilled 95% EtOH	Redistilled 95% EtOH	Distilled H$_2$O	2	5.0	15	0.2	0.2	0.2	0.6

Worker No.	Reaction		Dilution		Instrument for reading color	Standard use	Per cent error with standard
	Temperature (°C.)	Time (min.)	Solvent	Amount (cc.)			
1	Room	60	None		Leitz colorimeter microcups	KMnO$_4$	0–20
2	25	60	95% EtOH	10	Ordinary colorimeter	Methyl red and cotton black	10–20 on urine extracts
3	Measured	60	None		Pulfrich photometer	Androsterone	1–3
4	Room	90	"		Hellige colorimeter	Glass	
5	25	60	Alcohol	10	Spekker photoelectric colorimeter	Androsterone	2
6	25	45	Redistilled 95% EtOH	10	Evelyn photoelectric colorimeter	"	2–4

* The numbers for the workers designate 1; Zimmermann (1); 2, Wu and Chou (5); 3, Zimmermann (6); 4, Oesting (9); 5, Callow et al. (7); 6, present paper.

was found to be superior to other KOH. This solution was checked by titration and manipulated if necessary to 5.00 N ± 0.02 N. The aqueous solution was adopted because it is much more stable than alcoholic KOH, the former lasting for a month, while the latter can be used for only 2 to 5 days. The blank is lower even at this high concentration. The only disadvantage is the possibility of carbonate precipitating out in high carbonate samples when the 10 cc. of EtOH are added. However, when protected by a paraffin seal, both the solid and the solution were found to be entirely satisfactory in this respect.

5 N concentration was adopted, since only this concentration gave as much color as did 2.5 N alcoholic KOH which was used in the beginning. Since as little as 20 γ per 0.2 cc. could be accurately measured, it was felt that this was a desirable KOH concentration. The KOH and the m-dinitrobenzene solutions were stored in the refrigerator.

Standard solutions of androsterone, dehydroisoandrosterone, and theelin. These were made from 2 mg. per cc. of stock solution by diluting 10 cc. to 100 cc.

Equipment—2 cc. microburettes which were calibrated to 0.01 cc., permitting the estimation of 0.001 cc., were used for measuring solutions. A De Khotinsky water bath was used for temperature control and an Evelyn photoelectric colorimeter for measuring the color intensity.

Procedure—Into the colorimeter tube were accurately measured 0.2 cc. of a test solution or 0.2 cc. of redistilled 95 per cent EtOH for blank, 0.2 cc. of m-dinitrobenzene solution, and 0.2 cc. of KOH solution. The tubes were then corked, gently shaken, and placed in a water bath for 45 minutes at 25° ± 0.2°, since this is a convenient laboratory temperature. At the end of 45 minutes the solution was diluted with 10.0 cc. of redistilled 95 per cent EtOH from a 10 cc. Koch burette. After the outside of the tube was dried and polished, it was placed in a rack for 3 minutes and read in the photoelectric colorimeter previously adjusted to 100 by means of the resistances with the blank in place.

Calculations—The concentration was calculated from the formula $C = L/K$, where L is obtained from an "L" table giving the relation of G readings to $2 - \log G$ and where K has been determined by standardization with known quantities.

We found, in agreement with Callow *et al.* (7), that a slight
loss of androsterone occurs when standard quantities are evapo-
rated to desired concentrations. A 45 minute reaction time was
adopted because the reaction is almost complete in 45 minutes
(Fig. 1).   It was felt that not enough increase in sensitivity was
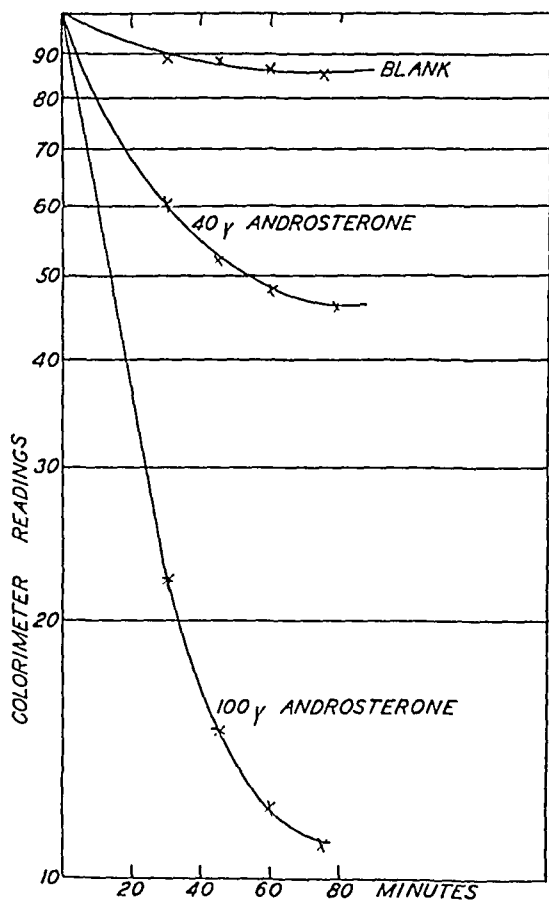
FIG. 1. Photoelectric colorimeter readings obtained with androsterone

gained by waiting until 60 minutes to offset the additional time.
The temperature was controlled within ±0.2°, because from
a temperature study the color production was found to vary
one division per degree.

    The pink color resulting from the reaction of *m*-dinitrobenzene
and androsterone in alkaline solution was found to increase upon

dilution for 2 minutes and to fade after 20 minutes (Table II). Hence 3 minutes after dilution was selected as a desirable time for reading.

Standardization—It was necessary to standardize each new lot of solid KOH, since the constant varied with different samples, and also to check for carbonate precipitation by adding 10.4 cc. of 95 per cent redistilled EtOH to 0.2 cc. of 5 N aqueous solution. Another lot of KOH should be used in case this test does not remain perfectly clear.

TABLE II

Measurement of Androsterone-m-Dinitrobenzene Color Stability after Dilution

The exponent figures designate fourths, thus $91^1 = 91\frac{1}{4}$.

Blank			40 γ per 0.2 cc.		
Min.	Sec.	Reading	Min.	Sec.	Reading
			0	0	Dilute
				5	65^0
0	0	Dilute		20	58^0
	5	91^1		40	54^2
	20	89^1	1	10	52^2
	44	88^0		55	51^2
1	25	87^1	2	55	50^3
2	15	87^1	4		50^3
3		87^0	7		51^1
6		86^3			
24		87^2	26		52^1

To guard against errors due to a variation in the constant, a standard was always run in parallel with the ten to fifteen samples in triplicate. Four concentrations, usually 20, 40, 60, and 80 γ of androsterone per 0.2 cc., were run under identical conditions, thus eliminating the possibility of error due to deterioration, contamination, or evaporation of reagents. During the entire work which involved the use of several different samples of KOH and a number of disturbing factors such as evaporation of standard, carbonate interference, etc., the constant was found to vary 13 per cent. For a single set of determinations in which standards were run the error was at no time greater than 4 per cent and usually approximately 2 per cent as shown by triplicate experi-

ments. The data in Table III show the variations of constants from day to day with the same KOH solution.

The average of these three constants is 71.3 ± 0.6 per cent. A comparison of the standard curves of androsterone, dehydroisoandrosterone, and theelin is given in Fig. 2. The results of this careful concentration study in contrast to the reversed results of the rough absorption spectra would indicate that the curves and therefore the constants are similar but not identical. Even

TABLE III

Variations in K Values on Different Days for Various Concentrations of Androsterone

The exponent figures designate fourths.

γ per 0.2 cc.	Final concentration	Reading	L	K	Reading	L	K	Reading	L	K
	mg. per cc.									
20	0.00189	73²	0.1337	70.6	72²	0.1397	73.9	72³	0.1382	73.2
28	0.00264	65⁰	0.1871	70.9	65¹	0.1855	70.4	65⁰	0.1871	71.0
40	0.00378	54⁰	0.2676	70.9	53¹	0.2736	72.4	53³	0.2696	71.4
40	0.00378	54⁰	0.2676	70.9	53⁰	0.2756	72.8	53³	0.2696	71.4
48	0.00454	47³	0.321	70.9	47¹	0.325	71.6	47¹	0.325	71.6
56	0.00528	42²	0.372	70.5	42⁰	0.377	71.5	42¹	0.374	70.9
60	0.00566	39²	0.403	71.1	39¹	0.406	71.6	39³	0.401	70.8
72	0.00679	33¹	0.478	70.5	32²	0.488	72.0	32²	0.488	72.0
80	0.00755	29²	0.530	70.2	29⁰	0.538	71.2	29⁰	0.538	71.2
Average............				70.7			71.9			71.5
				±0.6%			±2.4%			±2.0%

when calculated on a molecular basis, the values are not within experimental error, particularly in the case of theelin which differs from the androsterone constant on this basis by 9 per cent. In so far as this is true, our results differ from the finding of Langstroth and Talbot (10) who claim the values of androsterone and dehydroisoandrosterone fall within their experimental error. Our result receives theoretical support from Weise and Tropp (4) who list the double bond as a supplementary activator of the methylene group.

Specificity—The great danger with colorimetric procedures is

that other substances may react to give color in addition to the substance we wish to measure. The statement is especially true of this reaction, as was stressed by Zimmermann (6) and Callow *et al.* (7) and as is shown by the following data. Of the forty-one compounds and three extracts investigated, those having a light transmission between 95 and 100 per cent for all wave-lengths, and hence can be considered as negligible or contributing no color, are stilbestrol, pregnanediol, benzoic acid, Δ^5-cholestene-
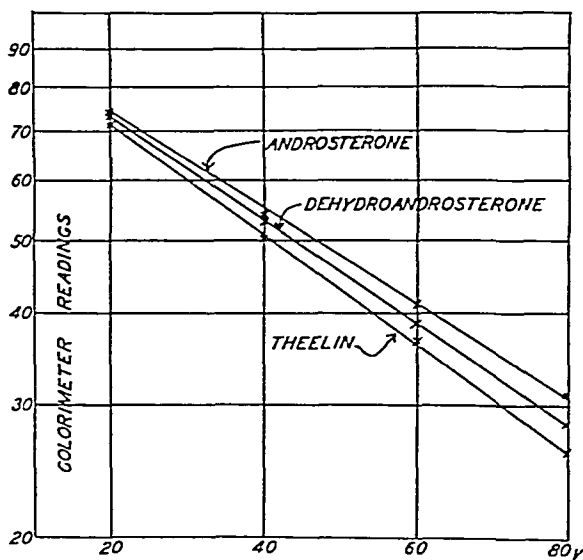
Fig. 2. Standard curves of androsterone, dehydroandrosterone, and theelin.

3,4-dione, α-cholesterol oxide, Δ^5-7-keto-3-hydroxycholenic ethyl ester, Δ^5-3,4-cholestenedione-4-enol acetate, Δ^5-3-acetoxy-7-keto-cholenic ethyl ester, Δ^5-3-acetoxy-5-hydroxy-6-ketocholenic ethyl ester, cholesterol, digitonin, β-hydroxybutyric acid, ergosterol, theelol, and 7-oxocholesterol. Those developing more color are given in Table IV. Several compounds are shown graphically in Fig. 3 in order to show the similarity of the rough absorption spectra of 17-ketosteroids and that of the urine extracts. The concentration used in all cases is 40 γ per 0.2 cc.

From these results it is obvious that the reaction is not specific for androsterone, nor is it entirely specific for 17-ketosteroids as Callow *et al.* (7) seem to be well aware. However, since of all

TABLE IV

*Compounds Contributing Significant Amount of Color in Colorimetric Androsterone Determination**

The exponent figures designate fourths.

Substance	Filter No.						
	660	635	620	565	540	520	420
Blank	92^3	92^0	91^1	88^1	87^0	86^0	78^0
Acetone	61^1	55^1	51^3	40^1	36^3	33^3	40^1
Androstenedione	83^2	73^2	66^2	46^0	42^1	38^0	49^2
Androstenetrione	82^0	72^1	66^2	48^0	44^1	40^0	51^0
Theelin	94^1	87^2	82^1	62^1	57^3	52^3	66^2
Androsterone	93^2	87^1	82^1	63^0	58^1	53^1	66^3
Dehydroisoandrosterone	95^2	89^1	83^3	64^0	59^1	54^3	71^2
Testosterone	90^2	86^1	83^3	77^2	76^2	74^1	73^2
6-Oxocholesterol	89^3	87^0	85^2	81^1	81^0	79^0	77^2
Δ⁴-3,6-Cholestenedione-6-enol ethyl ether	81^0	72^0	69^0	67^0	68^0	67^3	78^2
Kendall, Compound A	95^1	93^2	92^3	89^1	89^0	87^2	86^0
" " B	93^3	91^3	90^3	86^1	85^1	82^3	76^2
" " E	90^2	86^3	85^0	79^0	78^3	74^1	72^1
Cholestanone	96^0	94^0	93^0	87^2	87^1	85^1	79^0
Cholestenone	92^2	89^2	87^3	83^1	83^0	81^0	80^2
" acetate	93^2	91^0	89^3	85^2	85^3	83^2	83^1
Epiallopregnanolone	97^3	98^0	97^1	95^3	93^3	92^2	92^3
3-Keto-12-hydroxycholanic acid	97^1	96^3	96^2	95^3	95^0	93^3	89^1
" ethyl. ester	97^2	96^2	96^2	95^1	95^0	94^2	89^0
Acetoacetic ester	94^2	92^2	91^1	82^0	77^2	72^0	65^0
6-Oxo-17-acetotestosterone	91^1	87^1	86^0	82^0	81^1	79^0	77^2
Ethyl ester keto acid	96^2	93^3	94^0	92^0	91^1	89^2	87^1
Dehydrocholic acid	96^0	94^3	94^1	90^3	89^1	87^2	85^1
Dehydrohyodesoxycholic acid	94^2	92^2	92^0	88^1	87^1	84^3	83^0
Desoxycorticosterone	91^2	87^1	85^2	79^1	77^2	74^2	74^2
Progesterone	88^1	83^0	79^3	70^3	68^1	65^2	65^3
Urine extract Pa-603-N	90^0	80^3	76^0	53^2	49^0	45^0	55^2
" " Pa-610-N	92^2	85^2	82^0	67^3	64^2	60^3	61^1
" " Exp-355-N	94^0	86^1	83^0	66^0	61^1	58^0	66^1

* We are indebted to Dr. Erwin Schwenk of the Schering Corporation for the progesterone and to Dr. G. F. Cartland of The Upjohn Company for the natural estrogens and to Dr. T. F. Gallagher for the bile acid esters.

the compounds examined, the 17-ketosteroids produce the most pronounced absorption in the green, we concur with them in terming the results of the method a measure of 17-ketosteroid, keeping in mind that other keto substances may contribute to a small extent also.
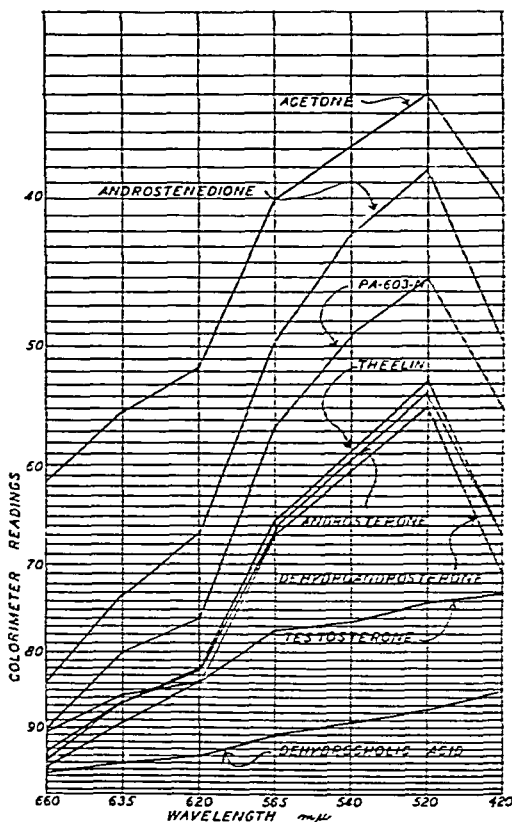
FIG. 3. Rough absorption spectra of several representative compounds combined with m-dinitrobenzene.

It is interesting to note in agreement with Callow *et al.* (7) that the absorption spectra of the urine extracts are similar to those of the 17-ketosteroids, which is good evidence that these substances are producing the color in urine extracts.

One unexpected finding was the green, rapidly fading color of

Δ^4-3,6-cholestenedione-6-enol ethyl ether in contrast to the usual fairly stable pink color produced by nearly all the other substances.

Application—While our main purpose was to investigate the application of the method to urine extracts, we found the method useful to determine the efficiency of alkali separation of pure androsterone from pure theelin, to show that the chromogenic property of pure dehydroisoandrosterone was not destroyed by boiling at pH 1 for 2 hours, to show that a significant amount of androsterone was *not* lost by the evaporation procedure involved in the alkali separation of the male and female hormones,

TABLE V

Androsterone Recovery from Urine Extracts Low in Androgen

Urine sample No.	17-Keto-steroid + androsterone added	17-Keto-steroid	Andros-terone recovered	Andros-terone added	Difference
	γ	γ	γ	γ	*per cent*
Pa-486-N................	71.6	34.6	37.0	38.5	−4
Pa-509-N................	80.8	43.2	37.6	38.5	−2
Pa-512-N................	51.0	11.0	40.0	38.5	+3
Pa-418-N*..............			38.2	38.5	−1
Exp-342-P*.............			37.4	38.5	−3
Average..					±3

* The color produced in these samples without androsterone addition was used as the blank.

and to determine the solubility of dehydroisoandrosterone digitonide.

Although most of the urine extracts are highly colored, the content of 17-ketosteroids is usually sufficiently high to require a dilution almost to eliminate the original red color of the extract. In spite of this a second blank was run to correct for this by adding 0.2 cc. of the diluted sample to 0.2 cc. of alkali plus 0.2 cc. of 95 per cent redistilled EtOH instead of the *m*-dinitrobenzene. The extent of this blank, not over one division with most samples, was added to the reagent blank.

It was found that commercial benzene contained sufficient chromogenic material to warrant its redistillation before use for extraction purposes. Since nearly all our samples had already

been biologically assayed, no correction could be made for this factor.

Recovery of androsterone added to urine extracts of low androgen content was well within experimental error as is shown in Table V.

In Table VI the values for thirty-six urine extracts are given which were routinely prepared and analyzed by the capon (8) and colorimetric methods. These figures are shown in a scatter diagram (Fig. 4).

The correlation of the present work is approximately one-half that of Oesting and Callow. Nevertheless, according to Wallace and Snedecor (11) a correlation coefficient with 35° of freedom and two variables such as we have, having a value of 0.42, is on the border of being highly significant, while the other values are extremely highly significant.

Another means of evaluating the results is to compare the variations of the ratio of the 17-ketosteroid method over the capon method.

	Minimum	Maximum
All urine extracts, present paper*	0.46	30.0
Eunuchoid, present paper	0.46	6.2
Callow *et al.* (7)	1.6	20.0
Oesting (9)	0.64	3.4

* Excluding samples where capon results show zero.

From this it can be seen that the variation of the ratio in the case of the present work is 5 times that of Callow *et al.* (7) and it is only when we consider eleven samples from the same patient that our extremes of variation compare in magnitude with those of Callow *et al.* (7). The reason for this difference is difficult for us to understand.

On the other hand, if we were to attempt to predict a value for one value from the results of the other, even in the case of the work of Callow *et al.* (7) and Oesting (9), this prediction could result in very large errors as can be seen from the scatter diagrams.

This is not difficult to account for when one remembers the non-specificity of the method; in fact, keeping this in mind one

TABLE VI

Results on Urine Extracts Analyzed by Capon and Colorimetric Methods

Sample No.	Sex	Classification	Treatment	Total volume	Collection	17-Ketosteroid (a)	Capon assay[a] (b)	Ratio $\frac{a}{b}$
				liters	*days*	*mg. per day*	*mg. per day*	
Pa-607-N	F.	Adrenal tumor	Postoperative	6.1	4	2.1	0.0	
Pa-618-N	"	Addison's disease	Desoxycorticosterone	5.4	4	2.2	0.0	
Pa-418-N	"	"	None	4.8	3	2.8	0.2	1.4
Pa-619-N	"	"	After desoxycorticosterone	11.7	5	3.5	0.2	1.8
Pa-617-N	"	"	None	13.7	5	3.5	0.8	4.4
Pa-512-N	"	Myxedema	"	4.5	4	5.7	0.0	
Pa-586-N†	M.	Eunuchoid	"	19.1	10	5.9	2.1	2.8
Exp-353-N	"	Normal	"	4.0	6	5.9	2.9	2.0
Pa-589-N†	"	Eunuchoid	Testosterone	7.9	6	7.1	3.0	2.4
Pa-606-N	"	Addison's disease	Desoxycorticosterone	5.8	5	7.8	5.7	1.4
Pa-610-N†	"	Eunuchoid	After testosterone	11.0	9	8.0	1.3	6.2
Pa-611-N	"	Addison's disease	None	10.6	5	8.2	3.0	2.7
Pa-486-N	F.	Amenorrhea and emaciation	"	4.1	8	9.0	0.3	30.0
Exp-356-N	M.	Normal	"	6.6	6	9.0	4.6	2.0
Exp-352-N	"	"	"	14.2	6	9.2	9.7	0.95
Pa-600-N	"	Addison's disease	"	12.3	8	9.3	10.4	0.9
Pa-588-N†	"	Eunuchoid	Testosterone	13.4	9	9.6	9.0	1.1
Exp-357-N	"	Normal	None	10.3	6	9.7	3.0	3.2
Pa-592-N†	"	Eunuchoid	Testosterone	5.6	5	9.7	5.8	1.7
Pa-609-N†	"	"	After testosterone	8.5	6	10.5	2.5	4.2

Pa-601-N	"	Pseudohermaphrodite	None	7.6	0	11.3	7.8	1.5
Pa-593-N†	"	Eunuchoid	Testosterone	15.0	9	11.9	7.1	1.7
Pa-603-N†	"		"	3.8	6	12.2	26.6	0.46
Pa-587-N	F.	Amenorrhea and dwarfism	None	5.5	6	13.1	7.0	1.9
Pa-605-N	M.	Addison's disease	"	9.1	4	13.4	5.2	2.5
Pa-597-N†	"	Eunuchoid	Testosterone	7.8	9	14.1	7.4	1.9
Pa-599-N†	"	"	"	6.0	6	14.1	8.6	1.6
Exp-354-N	"	Normal	None	5.2	6	14.2	7.4	1.9
Pa-509-N	F.	Hypertrichosis	"	15.3	6	15.0	0.6	25.0
Pa-598-N	M.	Addison's disease	After desoxycorticosterone	9.1	4	15.3	9.1	1.7
Pa-602-N	F.	Hirsutism, amenorrhea, obesity	None	4.0	6	15.6	15.0	1.0
Exp-355-N	M.	Normal	"	6.0	6	18.9	10.8	1.8
Pa-240-N	F.	"	"	2.7	2	21.7	4.6	4.7
Pa-239-N	"	"	"	3.2	2	23.0	4.5	5.1
Pa-604-N†	M.	Eunuchoid	Testosterone	0.5	9	25.3	17.3	1.5
Pa-210-N	F.	Normal	None	2.8	2	47.3	8.5	5.6

* These data as well as those of Callow et al. (7) and Oesting (9) have been treated statistically and the correlation coefficients found are: Callow et al. (7), r = 0.854; Oesting (9), r = 0.810; present paper, r = 0.419.

† Same patient.

would predict that the colorimetric method would result in higher values with urine extracts than the capon method, and, depending on the variation of the chromogenic and androgenic substances, a rather high variability or a low correlation would be expected.

Since we are most interested in getting a measure of testosterone metabolism in the body, it is our opinion that this can be done better by a consideration of the results of both methods than by either alone, since the colorimetric method includes androgenic
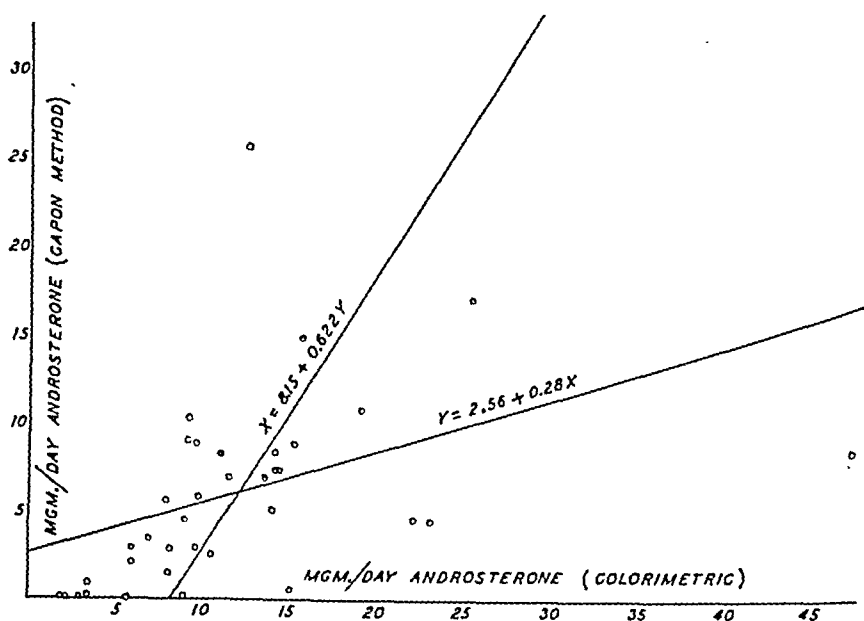
FIG. 4. Scatter diagram of the data obtained on urine extracts by the colorimetric and capon methods.

and non-androgenic chromogenic substances and very little or none of the non-chromogenic androgens but the capon assay includes androgenic chromogenic, and non-chromogenic androgenic materials, but none of the non-androgenic chromogenic substances. A combination of the two methods gives us a more reliable and complete picture of the metabolism of testosterone in the body.

That the colorimetric and capon results both rise in urine extracts from a eunuchoid receiving increasing doses of testosterone propionate is shown in Fig. 5. The ratios are, however, not of the same order throughout, ranging from 0.46 to 6.2. The very

striking exception is the excretion during the 6 day period when
50 mg. of testosterone propionate were administered per day.
In this case it is possible that some of the original testosterone
appeared in the urine. Either testosterone or some other very
potent androgenic substance of low or zero chromogenic content
must have been excreted.
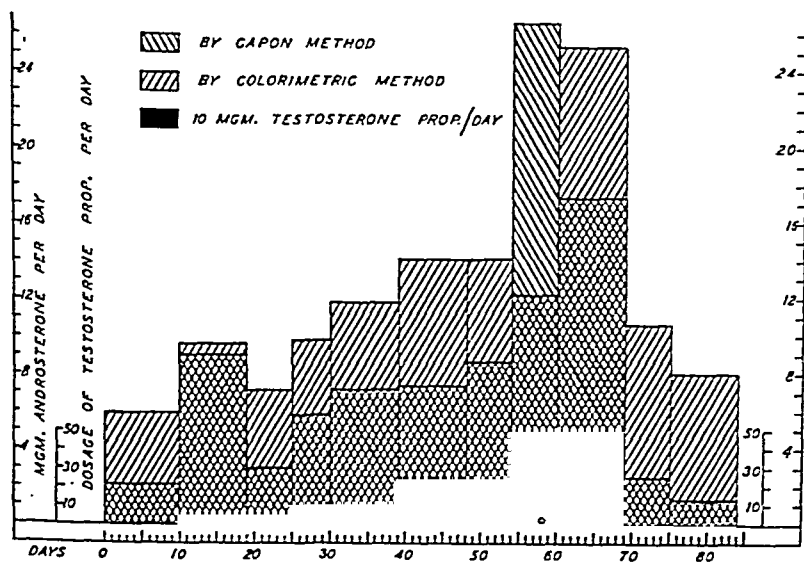
FIG. 5. Androgen excretion in a eunuchoid

SUMMARY

1. The Zimmermann method for the colorimetric estimation
of sex hormones has been modified, standardized, and adapted to
the Evelyn photoelectric colorimeter.

2. Of forty-one pure compounds examined, fifteen do not give
a color. The other twenty-six pure substances give enough color
at a 40 γ level to interfere seriously. However, the approximate
absorption spectra of these compounds over the 420 to 660 mμ
range show that acetone, androstenedione, theelin, androsterone,
dehydroisoandrosterone, and urine extracts are very similar in
nature with the peak at 520 mμ. The other reacting substances
give lower color values with the peak at 420 mμ.

3. The colorimetric method is of considerable value for testing methods for the recovery of pure androsterone and theelin, for solubility studies, and partition coefficients on pure compounds.

4. The results by the colorimetric method on urine extracts are almost always considerably higher than by the capon assay. In only one case was the order strikingly reversed. This was in a eunuchoid treated with very high doses of testosterone propionate.

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THE EFFECT OF VARIOUS MEDIA AND OF PYROPHOS-PHATE ON THE RESPIRATION OF LIVER TISSUE*

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In studies of the respiration of surviving tissues, a fundamental question is that of the nature of the medium to be used. The importance of this factor was stated by Thunberg (1) in 1909 and has since been reiterated frequently. Although it may be generalized that the "best" respiration fluid, other than actual blood serum, is that most closely approximating serum, the range of media used by various workers is indication that this is not always the case.

The following studies show that the optimal respiration of liver tissue does not necessarily occur in a so called physiological medium, and that all media are not equally satisfactory for demonstrating pyrophosphate inhibition.

Methods

All animals were normal male and female white rats of approximately 300 gm. weight. They were killed by a blow on the head, the blood drained off for a moment after the throat was cut, and the liver removed as rapidly as possible. If slices were to be cut, the liver was immediately placed on ice, and all bits of extraneous tissue and coagulated blood were removed. The slices were then cut and suspended for the few minutes until used in 0.9 per cent NaCl. The excess liquid was removed from the slices by being lightly blotted on filter paper, and the slices were then inserted into Warburg flasks.

* A preliminary report of this work was presented before the Ninety-sixth meeting of the American Chemical Society at Milwaukee, September 8, 1938.

When the tissue was to be minced, it was taken from the animal, rapidly cleaned of extraneous material, and minced with an ice-cold Latapie mincer. The mince was weighed on slips of cellophane on a torsion balance, inserted into the flasks, and the clump of tissue dispersed by means of a thin glass rod or metal needle.

Dry weights were determined for the slices by removing these at the end of the experiment, washing them well with distilled water, and finally drying them on small watch-glasses at 110°. Dry weights of the mince were determined by weighing out samples onto watch-glasses, mixing with a little distilled water, and similarly drying.

The respiration was measured by Warburg manometers at a temperature of 38.2°. The figures given are those actually obtained 60 or 90 minutes after the stop-cocks were closed, although readings were taken at 15 minute intervals. If the oxygen uptake was sufficient to make it necessary to renew the volume of air, this was done without making any allowance for the short time that the manometer was opened. Each pair of *Kästchen* flasks used for the work with bicarbonate solutions was calibrated to contain 2.0 and 10.0 cc., respectively. Warburg flasks used for other media contained 2.0 cc.

Media Used—Nine different solutions were used as respiration fluids. Of these, only the bicarbonate buffer contained glucose (0.2 per cent), and this was added in half the experiments. Since its presence had no effect on the normal oxygen uptake or the action of pyrophosphate, all bicarbonate experiments are listed together. The composition of the media is as follows:

Medium 1—Borate buffer. 2 cc. of 0.2 N NaOH + 1.2404 gm. of H_3BO_3 + 0.3432 gm. of KCl; all diluted to 200 cc.; pH 7.4.

Medium 2—M/60[1] Ringer-phosphate buffer (2). 102 parts of 0.9 per cent NaCl, 4 parts of 1.15 per cent KCl, 1 part of 3.82 per cent $MgSO_4$, 21 parts of a solution containing 40 cc. of 0.25 M Na_2HPO_4 + 2 cc. of N HCl diluted to 100 cc.; pH 7.4.

Medium 3—M/120 Ringer-phosphate buffer. Medium 2, diluted 1:1 with 0.9 per cent NaCl; pH 7.4.

Medium 4—0.05 M Ringer-phosphate buffer. Medium 2, but with only one-third the water (thus 6 times as concentrated as

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[1] Molarities are given in terms of phosphate concentrations.

Medium 3 with respect to K^+, Mg^{++}, Cl^-, SO_4^-, and $PO_4^=$); pH 7.4; strongly hypertonic.

Medium 5—0.1 M plain phosphate buffer. 17.9 gm. of $Na_2HPO_4 \cdot 12H_2O$ + 10 cc. of N HCl, all diluted to 500 cc.; pH 7.4.

Medium 6—0.9 per cent NaCl; unbuffered.

Medium 7—5.4 per cent NaCl; unbuffered; strongly hypertonic.

Medium 8—6.9 per cent KCl; unbuffered; strongly hypertonic.

Medium 9—Ringer-bicarbonate buffer. 100 parts of 0.9 per cent NaCl, 4 parts of 1.15 per cent KCl, 3 parts of 0.11 M $CaCl_2$, 1 part of 2.11 per cent KH_2PO_4, 1 part of 3.82 per cent $MgSO_4$, 21 parts of 1.3 per cent $NaHCO_3$; with or without added glucose to 0.2 per cent. Saturated before use with 5 per cent CO_2-95 per cent O_2 mixture; pH 7.4.

When Medium 1 was used, 1 cc. of it was diluted with 1 cc. of 0.9 per cent NaCl and used thus. If additions were made, they replaced part of the NaCl, so that the osmotic pressure, as well as the concentration of borate ion and KCl, remained approximately constant.

When Medium 2 was used, 2.0 cc. of it were put in the flask. If supplements were to be added, a more concentrated Ringer-phosphate buffer (as Medium 4) was used, diluted with substrate or inhibitor solution and with water. Thus the final concentrations of $PO_4^=$, Mg^{++}, and K^+ were kept the same as in the control flask.

Medium 3 was used by adding to the flasks 1 cc. of Medium 2 plus 1 cc. of 0.9 per cent NaCl. Supplements replaced part of the saline.

Medium 4 was used as such.

Medium 5 was prepared in double concentration and diluted with water. Added materials replaced part of the water, so that the phosphate concentration remained 0.1 M.

Medium 6 was used as such, and added materials simply replaced part of the solution.

Media 7 and 8 were used as such. The figure of 5.4 per cent was chosen because this is 6 times the isotonic concentration of NaCl, just as Medium 4 is 6 times the concentration of Medium 3. 6.9 per cent KCl is the molar equivalent of 5.4 per cent NaCl.

Medium 9 was used as given.

Pyrophosphate was added to all buffers but the bicarbonate as

0.1 M solution, pH 7.4, in such amounts as to give a final concentration in the respiration vessel of 0.03 M. With the bicarbonate buffer, one-tenth of the solution was replaced by 0.3 M pyrophosphate, pH 7.4, giving a final concentration of 0.03 M pyrophosphate.

Results

In Table I the results are expressed as U_{O_2} (c.mm. of O_2 taken up per mg. of dry weight of tissue per unit of time) values at 60 and 90 minutes.

TABLE I

Effect of Various Media and of Pyrophosphate on Oxygen Uptake of Minced and of Sliced Rat Liver

Oxygen uptake is reported as c.mm. per mg. of dry weight of tissue per given time; pyrophosphate at a final concentration of 0.03 M.

	Minced liver					Sliced liver				
	Control			Per cent inhibition by pyrophosphate		Control			Per cent inhibition by pyrophosphate	
	No. of experiments	60 min.	90 min.	60 min.	90 min.	No. of experiments	60 min.	90 min.	60 min.	90 min.
Borate..........	45	3.8	4.8	37	35	7	7.0	10.0	26	22
M/60 Ringer-phosphate.....	5	2.9	3.4	33	29	6	6.8	9.4	40	38
M/120 Ringer-phosphate.....	32	3.1	3.9	19	18	4	7.5	9.9	29	24
0.05 M Ringer-phosphate.....	5	2.3	2.9			2	3.1	4.2		
0.1 M plain phosphate..........	6	2.3	3.0	22	27	6	4.9	6.4	33	30
0.9 % NaCl.....	19	3.4	4.2	32	31	9	4.0	5.0	5	−4
5.4 % "	7	1.5	1.8			5	1.7	1.8		
6.9 % KCl.......	2	1.3	1.7			2	1.5	2.1		
Ringer-bicarbonate........	4	2.7	3.8	41	53	4	7.6	10.9	−22	−19

In the average results of the experiments with minced rat liver, it is observed that optimal respiration was obtained in the borate buffer, while unbuffered 0.9 per cent NaCl supported respiration somewhat better than the phosphate and bicarbonate buffers.

The hypertonic solutions used inhibited the respiration strongly. The effect of hypertonicity is greater than that of hypotonicity, as tissue (either minced or sliced) will respire in distilled water and give actual Q_{O_2} values greater than obtainable with any of these hypertonic solutions.   0.03 M pyrophosphate inhibits the respiration of *minced tissue* from 20 to 50 per cent, regardless of the medium used.

The results of the experiments with sliced liver show that the borate buffer, the Ringer-bicarbonate, and the M/60 and M/120 Ringer-phosphate buffers give about the same respiration, which is approximately twice as high as the best oxygen uptake obtained for the mince.   Plain physiological saline will not support the respiration of sliced liver as well as will the borate or the four Ringer buffers mentioned.   Hypertonic solutions decrease the respiration of sliced tissue as they did with the mince.

0.03 M pyrophosphate inhibits sliced liver in a borate buffer, in an M/60 or M/120 Ringer-phosphate, or in a plain 0.1 M phosphate buffer, from 20 to 40 per cent.   Pyrophosphate, however, exerts no inhibitory effect on slices respiring in plain physiological saline and actually stimulates respiration in a Ringer-bicarbonate buffer.   The lack of pyrophosphate inhibition in 0.9 per cent NaCl has been observed on samples of the same liver that was strongly inhibited by pyrophosphate when respiring in other media.

DISCUSSION

It is observed that our sole criterion for comparing the various media has been the oxygen uptake of liver mince and slices without added substrate.   The facts observed may not hold true for tissues reacting with added substrate or in which specific reactions are being observed, as for example phosphorylation.

Other workers have studied pyrophosphate inhibition.   Dixon and Elliott (3), using slices of rat liver in phosphate buffer, found a 30 to 40 per cent inhibition of respiration by M/30 pyrophosphate. Krebs and Eggleston (4), working with guinea pig liver in a 0.1 M phosphate buffer, pH 7.1, reported inhibition by 0.015 M pyrophosphate on minced tissue and no inhibition on sliced tissue. Greig and Munro (5), using rat liver slices respiring in a bicarbonate medium, found M/30 pyrophosphate to inhibit only 10 per cent, which they consider within the experimental error.   Our

observations have been that neutralized 0.03 M pyrophosphate will consistently inhibit either sliced or minced rat liver tissue, whether respiring in a borate buffer, a plain 0.1 M phosphate buffer, or an M/60 or M/120 Ringer-phosphate buffer, the inhibition ranging from 20 to 50 per cent. It will likewise inhibit, to the same degree, minced tissue respiring in unbuffered 0.9 per cent NaCl or in Ringer-bicarbonate buffer. It has no effect on sliced tissue respiring in 0.9 per cent saline, and in Ringer-bicarbonate it has been shown to stimulate the oxygen uptake of liver slices.

SUMMARY

In studying the respiration of minced and sliced rat liver tissue, we have found that:

1. In a borate buffer, minced tissue has an oxygen uptake greater than in any of eight other media tried. Slices in borate medium have an oxygen uptake as high as in any of the others, the values being approximately twice those for the greatest oxygen uptake of the minced tissue.

2. In unbuffered 0.9 per cent NaCl, mince respires as well as in any of the other media except the borate. 0.9 per cent NaCl will not, however, support oxygen uptake of slices as well as the borate, phosphate, or bicarbonate media.

3. Hypertonic solutions inhibit the oxygen uptake considerably, both of sliced and minced liver.

4. 0.03 M pyrophosphate will inhibit the respiration of minced and sliced tissue in all the media, with the exception of slices respiring in an unbuffered 0.9 per cent NaCl solution or in a Ringer-bicarbonate buffer. In the saline solution, pyrophosphate exhibits no effects, and in the Ringer-bicarbonate solution a stimulation of oxygen uptake by pyrophosphate is noted.

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SYNTHESIS OF ESTERS OF PHOSPHORIC ACID RELATED TO PHOSPHATIDES

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This paper reports the synthesis of esters of phosphoric acid related to the naturally occurring phosphatides. Aminoethylphosphoric acid resulted from the reaction of ethylenimine with phosphoric acid. The applicability of this cyclic imine to the preparation of esters related to cephalins was suggested by this reaction. The syntheses of cetylphosphoric acid, cetyl-β-chloroethylphosphoric acid, and β-aminoethylcetylphosphoric acid are described. The last compound has a structure analogous to that ascribed to natural cephalins, the cetyl radical serving as a model for the diglyceride portion of the cephalin molecule.

EXPERIMENTAL

Ethylenimine and Phosphoric Acid—260 mg. of ethylenimine (boiling at 55.5–55.9°) were cautiously added to 0.70 ml. (1.7 moles) of 85 per cent orthophosphoric acid. The mixture was heated at 105° for 24 hours, then taken up in water, and brought to pH 10 with barium hydroxide. Barium phosphate was centrifuged off and barium salts of phosphoric esters precipitated by adding 1.5 volumes of 95 per cent alcohol. The barium salt was redissolved in water and reprecipitated by alcohol. The free aminoethylphosphoric acid was isolated and purified by precipitation of the barium with dilute sulfuric acid and crystallization by addition of 4 parts of alcohol. Yield, 67 per cent on the basis of the ethylenimine; colorless needles.

$C_2H_8NO_4P$. Calculated, P 22.0, N 9.93; found, P 21.9, N 9.90
M. p. 240° with decomposition

Cetylphosphoric Acid—4.1 gm. of cetyl alcohol were dissolved in 10 ml. of carbon tetrachloride, 2.9 gm. of phosphoryl chloride added, and the solution refluxed 3 hours. The solvent was evaporated in a vacuum and the residue kneaded in a mortar with cold saturated barium hydroxide solution, replaced frequently until phenolphthalein in the aqueous phase remained colored through several minutes of agitation. The insoluble residue was washed once with alcohol and extracted exhaustively with benzene. It was then suspended in slightly more than an equivalent amount of half normal sulfuric acid, worked up until suspended, and centrifuged. The treatment was repeated with a similar volume of 0.1 N sulfuric acid. The insoluble material was then extracted with alcohol containing a little sulfuric acid. To the clear alcohol solution 10 volumes of 0.1 N sodium chloride solution were added slowly. The precipitated ester was filtered off by means of a Buchner funnel, sucked dry, and washed with an equal volume of cold acetone; it was then dried *in vacuo* and recrystallized from alcohol. Yield, about 70 per cent; m.p., 71°; P, calculated 9.64 per cent, found 9.60.

The method of separation employed by Plimmer and Burch (1) depended upon the water solubility of. barium cetyl phosphate, which we could not dissolve in water.

Cetylchloroethylphosphoric Acid—4.1 gm. of β-chloroethylphosphoryl dichloride (2) and 4.8 gm. of cetyl alcohol in carbon tetrachloride were refluxed 5 hours. The solvent was evaporated in a vacuum; the residue was transferred to a mortar and rubbed up energetically with cold saturated aqueous barium hydroxide, added gradually from a burette at such a rate that the water was kept barely pink to phenolphthalein. At frequent intervals, whenever the state of the pasty mass permitted decantation, the aqueous phase was replaced with pure water. When neutralization was complete, the insoluble mass was centrifuged down, washed twice with very dilute barium hydroxide solution, dried *in vacuo*, and then suspended in 250 ml. of anhydrous benzene. After cooling overnight at 5°, the barium salt was centrifuged off and extracted repeatedly with cold benzene. The crude barium cetylchloroethylphosphate, contaminated with barium carbonate, on the basis of its phosphorus content represented a 60 per cent yield.

The free cetylchloroethylphosphoric acid was separated by suspending the barium salt in a slightly more than equivalent volume of 0.3 N sulfuric acid. 4 volumes of alcohol were added and the insoluble matter centrifuged off and discarded after being twice extracted with absolute alcohol. The combined alcohol solutions were concentrated and the water partially replaced by alcohol by the addition of absolute alcohol and further concentration. At a volume of 50 ml. the acid was crystallized by dropwise addition of cold water. The compound was then recrystallized from acetone. White lustrous needles, very soluble in alcohol and benzene, were obtained.

Calculated, P 8.07, Cl 9.21; found, P 8.00, Cl 9.16; m.p. 54.5°

Aminoethylcetylphosphoric Acid—2 gm. of cetylchloroethylphosphoric acid were heated in a sealed glass tube with 50 ml. of an anhydrous, nearly saturated solution of ammonia in ethanol, at 110° for 48 hours. The solvent was evaporated *in vacuo* and the residue shaken up in acetone, which was then completely evaporated. The residue was suspended in 25 ml. of cold 95 per cent ethyl alcohol; after standing at 5° overnight the insoluble material was centrifuged down and recrystallized several times from 95 per cent alcohol. After three recrystallizations the yield was 24 per cent of the theoretical. White crystals, soluble in benzene, slightly soluble in acetone and alcohol, more so in 95 per cent alcohol, were obtained. M.p., 226°, corrected, with decomposition.

Calculated. C 59.2, H 11.05, N 3.84, P 8.50
Found.   " 59.8, " 11.20, " 3.79, " 8.42

SUMMARY

The syntheses of aminoethylphosphoric acid by the action of ethylenimine on phosphoric acid, and of cetylphosphoric acid, chloroethylcetylphosphoric acid, and aminoethylcetylphosphoric acid have been described. The last compound was prepared as an analogue of natural cephalins.

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PHYTIC ACID IN AVIAN ERYTHROCYTES

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In the course of studies on the distribution of phosphorus compounds in the blood of various species, diphosphoglyceric acid was found to make up about half of the organic acid-soluble phosphorus of most mammalian bloods. It was absent, however, in the bloods of all species examined which normally have nucleated erythrocytes. The high concentration of organic acid-soluble phosphorus in these blood cells indicated the presence of a considerable amount of a hitherto unidentified phosphoric ester. Subsequent investigation led to the identification of this unknown compound as inositolhexaphosphoric acid ester, commonly known as phytic acid. This substance accounts for approximately half of the organic acid-soluble phosphorus in the erythrocytes of chickens and several other birds, and seems to be present in turtle erythrocytes. Quantitative studies of this substance in the blood of various species will be reported later.

While phytic acid is known to occur ubiquitously in plants, this is the first time, to our knowledge, that it has been found in the animal kingdom.

EXPERIMENTAL

Preparation of Barium Phytate from Chicken Blood—Approximately 2 liters of chicken blood were collected and mixed with sodium citrate at the slaughter-house. The proteins were precipitated with 500 cc. of 40 per cent trichloroacetic acid, and 1500 cc. of water were added. After centrifugation the supernatant fluid was drawn off and filtered. To the filtrate were added 50 gm. of ammonium chloride and 50 gm. of magnesium nitrate dissolved in a small amount of water. The fluid was then neutral-

ized with concentrated ammonium hydroxide and an excess of 60 cc. of concentrated ammonium hydroxide was added. An abundant precipitate formed and settled out rapidly. After 2 hours, the supernatant fluid was decanted and the precipitate stirred up with a large amount of approximately 0.1 N ammonium hydroxide. After centrifugation and withdrawal of the supernatant fluid, the precipitate was dissolved in 200 cc. of N HCl. This solution was neutralized with NaOH,[1] and 1/6 volume of N HCl was added. A concentrated solution of ferric chloride was then added, until the supernatant fluid, above the creamy white precipitate, was yellow. Approximately 20 gm. of NaCl were then added and the mixture was kept in a boiling water bath 1 hour. The precipitate was centrifuged, separated from the supernatant fluid, and thoroughly stirred into a small volume (about 50 cc.) of distilled water. Sodium hydroxide was added in small portions until the iron hydroxide coagulated. The mixture was then heated in a boiling water bath to complete the decomposition. The precipitate of ferric hydroxide was separated by centrifugation and washed once with distilled water to which a few drops of 0.5 N NaOH had been added. The supernatant fluid and the washings were combined and acidified. Barium hydroxide was then added in sufficient amount to render the solution alkaline to phenolphthalein. The precipitate was dissolved in N/6 HCl and reprecipitated with barium hydroxide. It was then twice redissolved in 0.1 N hydrochloric acid and precipitated with 1.5 volumes of 96 per cent alcohol. The barium salt was washed successively with 50 per cent alcohol, 96 per cent alcohol, 1:1 alcohol-ether, and ether, and then dried at 120°. Analysis of this material gave the following results.

$(C_6H_{11}P_6O_{24})_2Ba_7$.   Calculated.   Ba 42.37, P 16.39
                           Found.   " 42.32, " 16.40
                                          " 41.89, " 16.42

[1] In other experiments a calcium precipitation was interposed at this point as follows: The neutralized solution was reacidified with an equal volume of N hydrochloric acid and heated for $\frac{1}{2}$ hour on the water bath to decompose all adenosine triphosphate. The solution was then mixed with an equal volume of 15 per cent solution of calcium acetate and the mixture kept at approximately 80° for 10 minutes to precipitate phytic acid. The precipitate was then dissolved in N hydrochloric acid, neutralized with sodium hydroxide, and the precipitation with ferric chloride carried out as described.

Isolation and Identification of Inositol—400 mg. of the barium salt, prepared as described above, were hydrolyzed for 90 hours with 14 cc. of 2 N H_2SO_4 in a boiling water bath. The sulfate and phosphate were removed with baryta. The excess of barium hydroxide was precipitated with carbon dioxide gas. The fluid was concentrated on the water bath and freed from a small amount of remaining barium carbonate by filtration. The filtrate was concentrated to 3 cc., 30 cc. of acetone were added, and the mixture put in the refrigerator. After 48 hours fine crystals had collected on the sides and bottom of the beaker. They were filtered and dried. The melting point of these crystals was found to be 221°; the melting point when mixed with a pure preparation of inositol was 222°. The Scherer reaction was positive. A benzoyl derivative gave a melting point of 258°, in accord with the value of Maquenne (1). The microanalysis (performed by Dr. V. Niederl) gave the following results.

Ash. 6.59, 6.36
Calculated on an ash-free basis. C 40.95, 40.69; H 6.09, 6.15
Theoretical. " 40.11 " 6.66

Although these analyses indicate that some impurities were present, they do not leave any reasonable doubt as to the identity of the compound.

Preparation of Sodium Phytate—Essentially the procedure of Posternak (2) was followed. After decomposition of the iron precipitate, 1 volume of alcohol was added to the filtrate and the solution put in the refrigerator. A semicrystalline syrup separated. After 2 days standing the supernatant fluid was decanted. The syrup was diluted with distilled water, filtered to remove remaining traces of iron hydroxide, and reprecipitated with alcohol. The alcohol precipitation was repeated three times and finally the syrup left to dry in a desiccator over phosphorus pentoxide. On analysis it was found that the salt had less water than is required by the formula of Posternak (2). Sodium analyses were made by Leva, according to his method (3).

$C_6H_6O_{24}P_6Na_{12} \cdot 21H_2O$. Calculated. Na 21.20, P 14.29, ash 61.29
Found. " 21.06, " 14.48, " 60.95

Preparation of Barium Salt from Turtle Blood—200 cc. of blood were collected in the laboratory from three sea-turtles. The

blood was deproteinized with trichloroacetic acid. Precipitation with magnesia mixture was carried out as described in the first section of the "Experimental" and then a calcium precipitation was made.[1] Finally, a barium salt was prepared. On this a single analysis gave the following results.

$$C_6H_{12}O_{24}P_6Ba_3 \cdot 3H_2O.$$ Calculated. Ba 36.8, P 16.61
Found. " 36.0, " 16.47

This analysis together with the typical properties of the salt observed during its preparation suggests strongly that phytic acid is present in turtle erythrocytes.

SUMMARY

Phytic acid has been isolated in large amounts from chicken blood. The same substance probably is present in turtle blood.

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COMPARATIVE CREATINE CONCENTRATION OF THREE VOLUNTARY MUSCLES IN SEVENTY-FOUR AUTOPSY CASES*

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Despite the wide interest that has been displayed in the rôle of creatine and phosphocreatine in muscle metabolism, comparatively few analyses have appeared upon the creatine content of human voluntary muscle. In the course of work being carried out upon creatine metabolism, it appeared important to obtain more extensive data on the creatine content of human voluntary muscle. The main object of the present study was to determine whether any significant differences existed between the creatine concentrations in different muscles of the body. In numerous studies in the literature the site of sampling has not been given. Accordingly, to attach any significance to figures shown, it is important to establish what variations, if any, exist between different muscles. It also appeared to be of interest to classify the findings according to the pathological diagnoses. Earlier studies from this laboratory (1–3) have summarized the literature bearing upon this subject, and it is unnecessary to recapitulate here.

EXPERIMENTAL

The methods of sampling and determining the creatine were those employed in previous studies from this laboratory (1, 2).

Creatine determinations were carried out upon 74 miscellaneous human autopsy cases. Three muscles were obtained from each, rectus abdominis, psoas major, and sternocleidomastoid.

TABLE I

Creatine Content of Human Voluntary Muscle

The results are expressed in mg. per 100 gm. of fresh tissue.

No. of cases	Muscle	Creatine			Condition of subject
		Average	Maximum	Minimum	
11	Pectoralis major (4)	443	462	422	Normal
51	" " (3)	405	538	212	Pathological
60	" " (1)	395	564	282	"
74	Rectus abdominis	405	576	133	"
74	Psoas major	402	614	159	"
74	Sternocleidomastoid	388	696	169	"

The figures in parentheses refer to bibliographic references.

TABLE II

Summary of Findings in Different Conditions

The results are expressed in mg. per 100 gm. of fresh tissue.

No. of cases	Rectus abdominis			Psoas major			Sternocleidomastoid			Diagnosis
	Average	Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	
8	423	559	316	429	544	300	467	552	253	Uremia with creatinine retention
19	425	544	299	415	426	291	388	595	207	Bronchopneumonia
9	443	576	236	432	525	301	426	696	216	Tuberculosis
6	389	490	284	385	559	253	365	495	306	Malignancy
9	323	421	133	309	389	159	334	451	226	Acute inflammation
13	412	466	332	396	508	250	389	635	196	Circulatory involvement
4	347	381	316	372	450	300	306	354	253	Uremia and heart failure
6	415	504	333	458	614	378	391	480	169	Miscellaneous

The average findings for the three muscles included in this series and for eleven normal pectoralis major muscles and two previous

series of analyses of the pectoralis major obtained from autopsy cases are shown in Table I. It will be seen that the creatine content of the psoas major and rectus abdominis and the pectoralis major in miscellaneous autopsy cases is very nearly the same, while that of the sternocleidomastoid is only slightly less. It is significant that throughout this series the last muscle nearly always grossly appeared to contain more connective tissue than the other muscles studied. The findings in this study therefore do not support the hypothesis that different skeletal muscles of the same species contain different and characteristic concentrations of creatine.

Table II contains a summary of the findings in various pathological conditions. It will be noted by comparing the average findings in the entire series with the findings in various conditions that relatively high values were encountered in cases of uremia with creatinine retention, bronchopneumonia, and tuberculosis, while relatively low values were obtained in cases of malignancy, acute inflammation, and uremia with heart failure.

<div align="center">DISCUSSION</div>

The findings on the three muscles studied in this series and upon the pectoralis major indicate that there is little difference in the creatine content of different voluntary muscles of the human. Such differences as have been found to exist in past studies based upon a limited number of analyses are believed to be due for the most part to variations in the relative amount of fat and connective tissue in the muscles, experimental error, and chance variations due to other causes not particularly attributable to any one muscle group. In the analyses reported by Bodansky (5), for instance, the diaphragm and intercostal muscles, which contain relatively more connective tissue, show the lowest values for creatine.

It should be noted that, while the average values show excellent agreement among the different muscles, large variations often occur between the different muscles in individual cases. While the cause of these variations was not always evident, it may be said that in many cases our observations led us to conclude that these differences were due to such factors as the relative fat, connective tissue, and water content of the muscles. Local conditions af-

fecting the nutrition of the muscles and the effect of activity may also have been contributing factors, although there was no apparent means of evaluating these factors.

In regard to the pathological findings, these studies indicate that many factors affecting the general health of the individual may be reflected in the muscle creatine. There is a definite tendency toward lowered muscle creatine values in cases in which the nutritional state of the muscles might be expected to be adversely affected. In conditions in which there is an associated fever or creatinine retention, and particularly where both of these factors are acting, the muscle creatine values appear to be considerably higher than in other pathological conditions. These problems have been discussed more fully in other communications (1-3).

SUMMARY

Comparisons were made of the creatine content of three separate muscles in 74 human autopsy cases to determine whether significant differences existed in different voluntary muscles. The average results were, for the rectus abdominis 405 mg., psoas major 402 mg., sternocleidomastoid 388 mg. per 100 gm. of muscle. These results agree well with other analyses of the pectoralis major. The slightly lower average value for the sternocleidomastoid is believed to be due to the higher connective tissue content of this muscle.

The findings have also been classified according to the pathological diagnoses.

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NORMAL CREATINE, PHOSPHORUS, AND POTASSIUM CONTENT OF HUMAN CARDIAC AND VOLUNTARY MUSCLE*

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While there are relatively few data in the literature dealing with the composition of cardiac and voluntary muscle obtained from human autopsy cases, there is a much greater scarcity of data upon individuals who have died sudden deaths and are therefore essentially normal. In the course of work being carried out by us on the variations in creatine, phosphorus, and potassium in cardiac and voluntary muscle we were able to obtain thirteen accident and homicide cases through the cooperation of Dr. Reuben Strauss and Miss Velma Hellerstein of Cuyahoga County Morgue.

Methods

Samples of the left and right ventricles and pectoralis major were obtained at autopsy and trimmed carefully to remove all visible fat, connective tissue, endocardium, epicardium, and large blood vessels. Handling of the tissue was carried out in a humidor to avoid loss of moisture. Approximately 2 gm. of tissue were transferred to a previously weighed, glass-stoppered Erlenmeyer flask containing 20 cc. of 2 N H_2SO_4. After weighing, the sample was autoclaved for 45 minutes at 15 pounds pressure, cooled, and diluted to 100 cc. Aliquots of this hydrolysate were taken for the determination of creatine and for ashings for phosphorus and potassium determinations.

Creatine was determined by the method previously used in this laboratory (1, 2). Phosphorus determinations were carried out by the method of Fiske and Subbarow (3) and potassium by the permanganate titration method of Kramer and Tisdall (4). Water and neutral fat determinations were carried out by the method of Hastings and Eichelberger (5).

TABLE I

Summary of Findings on Thirteen Normal Human Subjects at Autopsy

The results are expressed in mg. per 100 gm. of fresh tissue.

		Left ventricle	Right ventricle	Voluntary muscle
Creatine	Average	203	165	443
	Maximum	218	185	462
	Minimum	186	154	422
Phosphorus	Average	194	160	201
	Maximum	204	187	216
	Minimum	180	140	195
Potassium	Average	285	219	328
	Maximum	305	242	346
	Minimum	251	189	311
Water*	Average	80.8	82.3	76.2
	Maximum	81.9	83.8	76.8
	Minimum	79.7	80.5	75.5
Fat*	Average	0.35	1.28	1.68
	Maximum	0.91	2.36	3.23
	Minimum	0.17	0.47	0.12

* Average of eight cases in per cent.

Results

The results of creatine, phosphorus, and potassium determinations on thirteen normal subjects are summarized in Table I. In addition, analyses of water and neutral fat were carried out upon eight of these subjects and the average findings are included in Table I.

The average creatine content of the left ventricle is approximately the same as in two previous series of miscellaneous autopsies (2, 6). The average creatine content of the right ventricle is about 10 mg. higher than in these two studies of miscellaneous cases. The phosphorus and potassium values are slightly lower

than those reported by Wilkins and Cullen (7) for normal hearts, but, after correction to a constant water content, our values agree very well with their figures.

The average creatine content of the pectoralis major is 443 mg. per 100 gm. This indicates that our previous estimate (2) of 425 mg. as the probable saturation level of voluntary muscle is too low. The individual values ranged from 422 to 462 mg., and the mean value of 443 mg. is very close to that reported by Bodansky (8) for three normal pectoral major muscles. Our values for phosphorus and potassium are similar to others reported in the literature (9).

The water content of the right ventricle was higher than in the left ventricle in all but one case, and, after correction for fat, it was higher in the right ventricle in every case. The fat content of the left ventricle was lower and was quite constant, while that of the right ventricle and pectoralis major was more variable.

DISCUSSION

The values for creatine, phosphorus, and potassium obtained in normal subjects are much more constant than in cardiac and skeletal muscle in miscellaneous pathological conditions. Perhaps the most significant finding in this series was the uniformly high creatine values for voluntary muscle. They suggest that there is some tendency toward low values in nearly all pathological conditions except those associated with fever and creatinine retention, where they may be elevated. However, one should not lose sight of the fact that it is difficult to compare results from persons who were quite active up to the time of death with those from patients who have been inactive for some time. The relatively younger age and other factors may have had some influence upon the findings.

Calculation of the creatine, phosphorus, and potassium content of the two ventricles on a wet and dry, fat-free basis revealed that a considerable part of the difference between the two ventricles could be attributed to the difference in water and fat content of the two ventricles. With the left ventricle as the standard of reference, the per cent difference was found to be for creatine 16.2, phosphorus 15.6, and potassium 21.8 on a fresh weight basis, and creatine 4.4, phosphorus 3.4, and potassium 10.8 on a dry,

fat-free basis.   Since the completion of this study, we have found that the right ventricle of the human heart contains more collagen than the left ventricle, suggesting that the small difference remaining is due largely to connective tissue.   Thus there is no basis for assuming any fundamental chemical differences between the muscle cells of the two ventricles.

There appears to be some correlation between the heart weight and concentration of the three constituents.   In general, there is a tendency toward higher values in both ventricles as the heart weight increases.   When one compares the creatine content of the two ventricles in the normal cases with the values obtained in a variety of pathological conditions, it becomes evident that low values are seldom encountered in the heart except when the heart itself becomes diseased.   Apparently the heart tends to maintain its creatine content at a high level and only loses it when the heart is failing.

SUMMARY

Analyses of the left and right ventricles and pectoralis major in thirteen human cases of sudden death gave average values of 203, 165, and 443 mg. of creatine, 194, 160, and 201 mg. of phosphorus, and 285, 219, and 328 mg. of potassium per 100 gm. respectively.   The results are discussed in relation to similar analyses on subjects with pathological conditions.

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THE CONVERSION OF PHENYLALANINE TO TYROSINE IN NORMAL RATS*

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The close structural similarity between phenylalanine and tyrosine had early suggested their metabolic relation. Embden and Baldes (1) have studied the interconversion of these amino acids by perfusing livers with dl-phenylalanine and subsequently isolating l-tyrosine from the perfusion fluid. They assumed that this conversion formed the main pathway in the oxidative degradation of phenylalanine; but Shambaugh, Lewis, and Tourtellotte (2) later brought forward evidence against this concept. Dakin (3) assumed that phenylalanine could be converted to tyrosine but described experiments indicating that other pathways of phenylalanine degradation were possible in the intact animal. There exists definite evidence that the animal organism can oxidize the phenyl group of phenylalanine to phenolic derivatives. Kotake et al. (4) observed the appearance of p-hydroxyphenylpyruvic acid in the urine of rabbits receiving large amounts of phenylalanine, while Medes (5) found that a patient suffering from tyrosinosis excreted an increased amount of tyrosine and p-hydroxyphenylpyruvic acid on administration of phenylalanine. The excretion of homogentisic acid by rats after administration of large doses of phenylalanine (6) and by guinea pigs and humans after ingestion of tyrosine (7) seems to indicate that both amino acids travel similar pathways during degradation. Since phenylalanine, in contrast to tyrosine, is an essential amino acid (8), the biological conversion of phenylalanine to tyrosine is probably an irreversible process.

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It was realized that the isotope method might show the extent to which the reaction occurs in normal animals and might also indicate the factors which influence it. We have prepared *dl*-phenylalanine containing stably bound deuterium, which was fed to normal rats as an addition to the stock diet. The tyrosine isolated from the proteins of the animals contained an amount of deuterium indicative of the origin of a large proportion of this amino acid from the dietary phenylalanine.[1]

Preparation of Deutero Phenylalanine and the Location of Deuterium in the Molecule—The deutero phenylalanine was prepared by heating non-isotopic *dl*-phenylalanine with 84 to 86 per cent deutero sulfuric acid, a method suggested by the work of Ingold *et al.* (9) who demonstrated that the hydrogen of some benzenoid substances becomes exchangeable under these conditions. The deuterium so introduced was "stably" bound, the isotope content being completely unchanged by boiling for long periods with 6 N hydrochloric acid in ordinary water; *i.e.*, under conditions of protein hydrolysis. This finding confirms previous results (10), which indicated that all hydrogen atoms in phenylalanine except those of the carboxyl and amino groups are stably bound.

It was expected from this method of preparation that most of the deuterium would be present in the benzene ring, and confirmation was sought through a mild degradation of the amino acid. The deutero phenylalanine was oxidized with chloramine T to benzyl cyanide and the latter hydrolyzed to phenylacetic acid. This conversion removes any deuterium present in the α position of the amino acid. The known reactions of phenylacetic acid and of its ethyl ester (11) suggested that the hydrogens of its methylene group in contrast to those of phenylalanine were "semilabile" and would exchange with the normal hydrogen of the solvent under the conditions of nitrile hydrolysis (Formula I). If this were true, the degradation of deutero phenylalanine to phenylacetic acid would remove any isotope present in the side chain of the

[1] While this work was in progress Dr. W. C. Rose kindly informed the authors that he was carrying out experiments on the conversion of phenylalanine to tyrosine, employing a deutero phenylalanine preparation obtained by a different process. The results of these independent experiments were similar to those reported in this paper.

amino acid. We have confirmed the lability of the hydrogen atoms in the methylene group of phenylacetic acid by hydrolysis of non-isotopic benzyl cyanide in heavy water and also by refluxing normal phenylacetic acid with hydrochloric acid in heavy water. In both cases the recovered phenylacetic acid contained nearly 2 atoms of non-ionizable deuterium per molecule.

The deutero *dl*-phenylalanine contained 16.8 ± 0.16 atom per cent deuterium, while the derived phenylacetic acid contained 22.2 ± 0.22 atom per cent, all of which must have been present
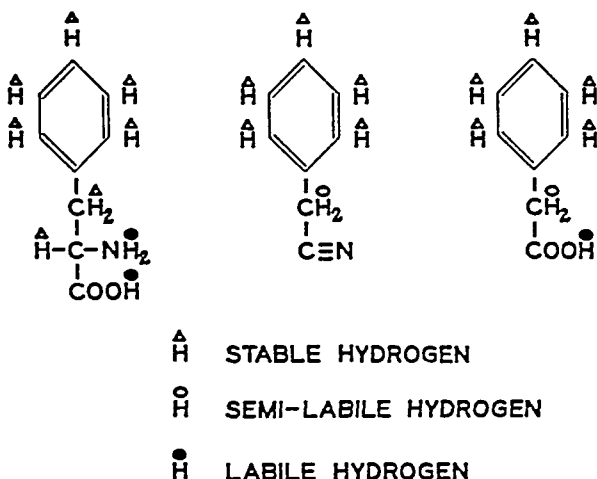
I. Changes in stability of hydrogen atoms during degradation of phenylalanine to phenylacetic acid.

in the benzene ring of the amino acid. If all the deuterium in the phenylalanine had been present in the benzene ring, the phenylacetic acid would have contained 23.1 atom per cent deuterium, while, if the isotope had been equally distributed over the eight stable positions of the amino acid, the degradation product would have contained only 14.4 atom per cent deuterium. Finally, if the deuterium had been equally distributed over the five ring positions and the α position, the phenylacetic acid would have contained 19.2 atom per cent of the isotope. It is evident therefore that almost all deuterium in the phenylalanine was located in the

phenyl ring, and that much less than 1 atom of isotope per molecule was present in the side chain.[2]

Calculation from the deuterium content of the phenylalanine and from the deuterium content of the sulfuric acid mixture shows that at least 5 of the 8 stable hydrogen atoms must have exchanged during preparation of the deutero amino acid. When taken in conjunction with the degradation results, this indicates very strongly that deuterium *entered all five ring positions*, probably to an equal extent.

Biological Experiments—In the first experiment a group of three immature rats having an average weight of 70 gm. was maintained for a 10 day period on our stock diet (13) to which 200 mg. of deutero *dl*-phenylalanine per rat per day were added. The animals showed an average gain in weight of 30 gm. during the period. At the end of the feeding period the animals were killed and two samples of tyrosine isolated, one from the protein of the combined internal organs and one from the protein of the remaining tissues; consisting mainly of muscle and skin. (The deuterium analyses are given in Table I.)

The experiment was repeated with adult rats having an average weight of 300 gm. which remained constant over the experimental period. They received an addition of 150 mg. of deutero *dl*-phenylalanine per rat per day. The tyrosine from the internal organ protein of this group contained only slightly less isotope than that of the first experiment (1.48 per cent), while the tyrosine of the carcass protein contained markedly less (0.47 per cent), a difference which may be ascribed to an increase in the amount of muscle and skin tissue in Group I. This process of protein synthesis was naturally absent, or almost absent in Group II.

The result of the experiment with adult animals that had received an appreciable amount of tyrosine in the casein of the stock diet

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[2] Two facts indicate, though they do not prove, that the small amounts of deuterium of the side chain were present in the α position. First, a preliminary experiment with *l*-phenylalanine showed that some racemization occurs when this amino acid is heated to 50° with 86 per cent sulfuric acid; and secondly, earlier experiments with fatty acids (12) and with aliphatic amino acids (unpublished observations) have shown that of the carbon-bound hydrogen atoms, only those α to the carboxyl group exchange under these conditions.

suggested that the conversion of phenylalanine to tyrosine represents one of the continuous processes which proceed independently of the animal's requirements for the reaction product. In a third experiment we therefore added to the stock diet of normal adult rats 150 mg. per rat per day of *l*-tyrosine, in addition to a deutero *dl*-phenylalanine supplement of 150 mg. per rat per day. Analyses (given in Table I) of tyrosine isolated from this group of animals indicate that *despite the large intake of tyrosine the animals still form considerable quantities of the amino acid from phenylalanine.*

TABLE I

Biological Conversion of Phenylalanine to Tyrosine by Rats

The *dl*-phenylalanine added to the diet contained 16.8 atom per cent deuterium.

Group No.	No. of animals	Average weight of animals	Amount of deutero phenyl-alanine added per rat per day	Deuterium content of tyrosine isolated from		Fraction of tissue tyrosine derived from added phenylalanine*	
				Internal organs	Rest of body tissues	Internal organs	Rest of body tissues
		gm.	*mg.*	*atom per cent*	*atom per cent*	*per cent*	*per cent*
I	3	70	200	1.96 ± 0.02	1.54 ± 0.02	27.6	21.6
II	2	300	150	1.48 ± 0.02	0.47 ± 0.02	20.8	6.6
III	3	330	150†	0.94 ± 0.02	0.34 ± 0.02	13.2	4.7

* These values were calculated on the assumption that tyrosine derived from the added phenylalanine contained 7.1 atom per cent deuterium.

† Plus 150 mg. of *l*-tyrosine.

The results show that phenylalanine is rapidly converted into tyrosine not only by growing, but also by full grown rats of constant body weight in which the total amounts of protein were maintained constant within narrow limits.

The isotopic tyrosine isolated from the proteins must have replaced tyrosine present in peptide linkage, a process requiring the opening of at least two peptide linkages per molecule of amino acid. A similar replacement process has been observed when isotopic tyrosine was added to the stock diet (13). The new results, in conjunction with former findings, demonstrate that such replacement is independent of the particular source of the amino acid, whether it is of dietary or of metabolic origin. This is new

support for the contention that in the living organism the amino acids of the proteins are continuously liberated and the vacant spaces filled by the same type of amino acid, originating from various sources (14, 15).

<div style="text-align:center">EXPERIMENTAL</div>

<div style="text-align:center">*Preparation and Properties of Deutero Phenylalanine*</div>

Action of Deutero Sulfuric Acid on Phenylalanine—The behavior of *l*-phenylalanine toward this reagent was first investigated in order to determine whether it was possible to obtain a fully optically active deutero amino acid by this means. 1 gm. of $l(-)$-phenylalanine ($\alpha_D^{20} = -35.0°$; 1.8 per cent in water) (Hoffmann-La Roche) was dissolved in 8.5 gm. of 86 per cent deutero sulfuric acid (16) containing 52 atom per cent deuterium and the solution maintained at a temperature of 50° for 3 days. After dilution with water the sulfate ion was precipitated with barium hydroxide and the filtrate evaporated to dryness *in vacuo*. The white solid was benzoylated with benzoyl chloride in sodium carbonate solution and the product crystallized from 25 per cent alcohol. Two distinct fractions were obtained: (1) a small amount of plate crystals which were identified by melting point and mixed melting point as benzoyl-*dl*-phenylalanine, and (2) a much larger amount of a more soluble material separating in tufts of long fine needles, m.p. 140–141.5°, $\alpha_D^{20} = +14.1°$ in M sodium hydroxide solution. This was undoubtedly benzoyl-*l*-phenylalanine containing about 7 to 8 per cent of the *d* isomer. It contained 12.03 ± 0.12 atom per cent deuterium, corresponding to 16.4 atom per cent in the parent phenylalanine or an average of 4.2 atoms per molecule. The results indicate that at least 20 per cent of the compound underwent racemization during the heating period.

The *dl*-phenylalanine used in the following reactions was prepared from α-acetaminocinnamic acid (17) by reduction with hydrogen iodide according to the procedure used by Harington and McCartney for the reduction of α-benzoylaminocinnamic acid (18).

2 gm. of *dl*-phenylalanine were dissolved in 8.5 gm. of 84 per cent sulfuric acid containing 50 atom per cent deuterium. The solution was maintained at 50° over a period of 6 days. The

freshly prepared solution was a pale brown color and this did not deepen during the heating period. Benzoyl-*dl*-phenylalanine was prepared from the sulfate-free filtrate in a yield of 70 per cent, m.p. 185–186.5°. It contained 10.94 ± 0.10 atom per cent deuterium, indicating a value of 14.9 atom per cent in the phenylalanine or an average of 4.7 deuterium atoms per molecule.

To investigate the stability of the introduced deuterium this benzoyl compound was hydrolyzed by boiling with 6 N hydrochloric acid under a reflux for 48 hours. After removal of hydrochloric acid and most of the benzoic acid by evaporation *in vacuo*, the residue was rebenzoylated. The isolated benzoyl-*dl*-phenylalanine contained 10.85 ± 0.10 atom per cent deuterium; *i.e.*, no deuterium was removed during the hydrolysis.

In other experiments *dl*-phenylalanine was treated with 86 per cent deutero sulfuric acid at 20–25° for 5 days and at 100° for 2 days. In the former case the benzoyl compound was isolated in a yield of 72 per cent and contained 5.60 ± 0.05 atom per cent deuterium. In the latter reaction a considerable amount of sulfonation apparently had occurred, for, in contrast to the other experiments, the sulfate-free filtrate was strongly acid and benzoyl-*dl*-phenylalanine was obtained in a yield of only 27 per cent. It contained 9.6 ± 0.09 atom per cent deuterium.

Preparation of Deutero dl-Phenylalanine—30 gm. of *dl*-phenylalanine were dissolved in 140 gm. of 84 per cent deutero sulfuric acid containing 54.5 atom per cent deuterium. The solution, enclosed in a flask terminating in a long, fine, open capillary, was maintained at 50° for 8½ days. At the end of this period the light pinkish brown-colored solution was diluted with 550 cc. of water and brought to pH 5.5 by dropwise addition of 9 N sodium hydroxide solution. During neutralization vigorous mechanical stirring was employed, and the temperature was maintained between 26–29°, thus preventing the separation of sodium sulfate. The deutero phenylalanine precipitated suddenly when the pH was approaching 5 as white, apparently amorphous, small particles; and after standing for half an hour at 28°, it was filtered off and washed with two 100 cc. portions of ice-cold water. The solid was twice crystallized from water after treatment with a very small amount of norit. The lustrous, colorless plate crystals, after drying *in vacuo* over phosphorus pentoxide, weighed 14 gm.

The mother liquors yielded a further 3.8 gm. of material, and these two fractions were combined. On 50 mg. of material the Lassaigne test for sulfur was completely negative. The substance contained 16.8 ± 0.16 atom per cent deuterium, an average of 4.8 atoms of deuterium per molecule of phenylalanine.

Analysis—Calculated for phenylalanine containing 16.8 atom per cent deuterium, C 64.7, H 5.5, N 8.4; found, C 64.5, H 5.4, N 8.4.

The final mother liquors from the crystallization and the sodium sulfate-containing mother liquors from the isoelectric precipitation were combined, heated to about 60°, and 9 gm. of copper acetate dissolved in water were added. The light blue phenylalanine copper salt was washed and dried *in vacuo* over phosphorus pentoxide. The yield was 11.7 gm. The total recovery of phenylalanine was thus 92 per cent of the starting material.

Degradation of Deutero Phenylalanine to Deutero Phenylacetic Acid—The degradation was first performed by dissolving *dl*-phenylalanine with 2 moles of chloramine T in water and allowing the oxidation to take place in this medium at 38° (19), but it was found that the main product under these conditions was phenylacetaldehyde.

As there was some evidence (20) that oxidation of amino acids to the nitrile by chloramine T was favored by an acid reaction, the process was carried out at pH 4.7. The buffer contained 168 gm. of citric acid and 353 gm. of sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) per liter.

1 gm. of deutero *dl*-phenylalanine was dissolved in 60 cc. of the buffer and 3 moles of chloramine T dissolved in 80 cc. of water added. The mixture was stirred mechanically and maintained at a temperature of 40° for 45 minutes. At the end of this period the reaction mixture was subjected to a steam distillation. The distillate (about 100 cc.), containing large colorless, oily globules, was made approximately 6 N to hydrochloric acid and boiled under a reflux for 12 hours. The colorless hydrolysate was three times extracted with ether and the combined extracts dried with calcium sulfate. The solvent was removed by distillation and the residue dissolved in 30 cc. of water containing 0.8 gm. of sodium carbonate. Extraction of the alkaline solution with ether in a continuous type of extractor for 6 hours removed a small amount of neutral, brownish yellow, oily material which did not solidify

on standing. . The alkaline solution was acidified to Congo red with sulfuric acid and again extracted with ether for 6 hours. This second ether extract was evaporated and the residue crystallized from water after treatment with a small amount of norit; m.p. 76.5–77° (corrected). After a second crystallization from petroleum ether (b.p. 30–60°) the material weighed 450 mg. It contained 22.2 ± 0.22 atom per cent deuterium. After the mother liquors were worked up and the recovered material crystallized from petroleum ether, there were obtained a further 150 mg. of phenylacetic acid, m.p. 76–77° (corrected), making a total yield of 72 per cent of the theory.

In other degradation runs the oxidation period was increased up to a maximum of 12 hours, but without further increase in the yield of phenylacetic acid.

Stability of Carbon-Bound Hydrogen in Phenylacetic Acid—1 gm. of non-isotopic *dl*-phenylalanine was oxidized with chloramine T in the manner described above. The steam distillate was extracted with ether, and the light yellow oil remaining after evaporation of the ether was refluxed with 15 gm. of concentrated hydrochloric acid and 15 gm. of 6.4 per cent heavy water for 16 hours. Phenylacetic acid was recovered from the hydrolysate as described above. The material, after treatment with norit, was crystallized twice from water and once from petroleum ether (b.p. 30–60°). It had a melting point of 76.5–77° and contained 0.82 ± 0.02 atom per cent deuterium. Since the hydrolysis mixture contained 3.66 atom per cent deuterium, this represents 1.8 atoms of deuterium per molecule of phenylacetic acid.

1 gm. of pure phenylacetic acid was boiled under a reflux with 10 gm. of 6.4 per cent heavy water and 10 gm. of concentrated hydrochloric acid for 22 hours. The solution was extracted with ether, the ether extract dried over calcium sulfate, and the solvent distilled off. The colorless crystalline residue was twice crystallized from water. It had a melting point of 76.5° (corrected) and contained 0.89 ± 0.02 atom per cent deuterium, corresponding to 1.9 atoms of deuterium per molecule of phenylacetic acid.

Biological Experiment

The deutero *dl*-phenylalanine was dissolved in 1.5 moles of sodium carbonate solution and added to the powdered stock diet (13), the volume of solution being so adjusted that the mixture

could be worked up into a moderately stiff paste. In the third experiment the non-isotopic tyrosine was added to the stock diet as a suspension in the deutero phenylalanine solution, the smallness of the suspended particles making it possible to take accurate aliquots. The growing animals received 10 gm. and the adult animals 15 gm. of stock diet per rat per day. It was found that these quantities were completely consumed during a 24 hour period.

The procedure for the isolation of tyrosine was identical in the three groups of animals. The animals were killed with ether and the internal organs (consisting of liver, heart, kidneys, lungs, spleen, and intestinal tract) removed and pooled. The intestinal tracts were opened and thoroughly washed out. The internal organs and the carcass tissue were minced and extracted, first with trichloroacetic acid solution to remove non-protein nitrogen, and then with alcohol and ether to remove as much fat as possible. The extracted tissues were hydrolyzed by boiling with 6 N hydrochloric acid for 24 hours. After the removal of pigment and excess hydrochloric acid from the hydrolysates by the usual procedures, tyrosine was precipitated by adjusting the pH to 5.0 to 5.5 with sodium hydroxide solution. The tyrosine was crystallized five times from water, norit being employed in the first crystallization, while in the second and third non-isotopic *dl*-phenylalanine was added to the solution in a concentration of 100 mg. per cent in order to "wash out" (15) any deutero *l*-phenylalanine which might have contaminated the tyrosine. The isolated tyrosine was analyzed for deuterium by the falling drop technique (21). Carbon and hydrogen and Dumas nitrogen analyses were made on two of the tyrosine samples, and micro-Kjeldahl nitrogen analyses on the remaining four samples.

Group   I—Calculated.   C 59.6, H 6.1, N 7.7
           Found.   Internal organs, C 59.6, H 6.2,   N (Dumas) 7.8
                    Carcass,   " 59.5, " 6.1,   "   "   7.7
Group II—Found.   Internal organs, N (micro-Kjeldahl) 7.8
                    Carcass,   "   "   7.7
Group III—Found.   Internal organs, "   "   7.7
                    Carcass,   "   "   7.7

Body Fluids—Prior to the removal of the internal organs some water was distilled off from the opened body. Deuterium anal-

yses of this water gave, for Group I 0.05 ± 0.01 atom per cent, for Group II 0.02 ± 0.01 atom per cent, and for Group III 0.03 ± 0.01 atom per cent. These values probably represent deuterium set free during complete oxidative destruction of phenylalanine and tyrosine as well as that released in the interconversion of the two amino acids.

DISCUSSION

Fraction of Tissue Tyrosine Derived from Dietary Phenylalanine

This value may be calculated from the deuterium content of the phenylalanine of the diet and that of the isolated tyrosine. If the isotope content (in atom per cent) of a conversion product were one-tenth of that in the administered material, at least 10 per cent of the former must have been derived from the latter.

Loss of Deuterium during Conversion Process—This generalization, however, holds only if the total number of hydrogen atoms in both compounds is the same, and if no deuterium becomes displaced by substitution or exchanged for normal hydrogen during the conversion or during the isolation process. While the number of hydrogen atoms does not differ in the two compounds in question, the isotope content certainly changed during the conversion. The deuterium present in the para position of phenylalanine must have been removed during the biological introduction of the phenolic group. Furthermore any deuterium present in the two positions ortho to the phenolic group would be almost completely replaced by normal hydrogen during protein hydrolysis, as it has been shown (10) that these hydrogen atoms in tyrosine are semilabile, exchanging slowly when this compound is boiled in 20 per cent hydrochloric acid (Formula II).

A phenylalanine preparation which contained deuterium equally distributed over the five ring positions would therefore lose the isotope marker from three of these positions, yielding a tyrosine having only 40 per cent of the deuterium content (in atom per cent) of the mother substance. The deutero phenylalanine used in the experiments under discussion contained 96 per cent of its isotope in the phenyl ring, and this was probably uniformly distributed over the five positions. If all the tissue tyrosine had been derived from the deutero phenylalanine added to the diet, the isolated tyrosine would therefore have contained a maximum of 7.1 atom per cent deuterium. This maximal value has been

used as the basis for calculating the figures set out in the last two columns of Table I.

Sources of Non-Isotopic Tyrosine—In our experiments a considerable amount of non-isotopic tyrosine originating from at least three sources must have been introduced into the tissue proteins. The figures in the last two columns of Table I therefore represent only minimum values, and the fraction of tissue tyrosine originating from phenylalanine must have been higher.

(a) The dietary phenylalanine was a mixture of deutero *dl*-phenylalanine and non-isotopic *l*-phenylalanine[3, 4] present as a casein constituent. The latter must have been a source of non-
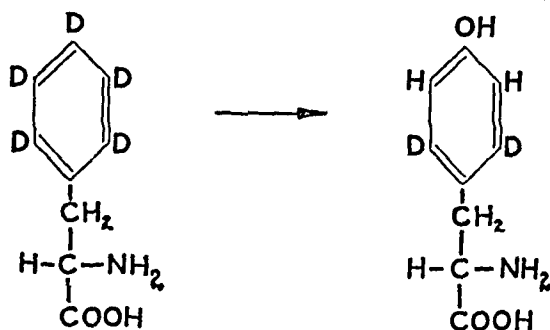
II. Conversion of deutero phenylalanine to deutero tyrosine in a medium of water.

isotopic tyrosine, and the amino acid formed from this mixture would have a deuterium content considerably lower than the value (7.1 atom per cent) employed in the calculation.

(b) The diet contained non-isotopic tyrosine[5] as a casein con-

[3] Casein contains 3.88 per cent of *l*-phenylalanine (8), so that the animals of Group I received 58 mg. and those of Groups II and III, 87 mg. of *l*-phenylalanine per rat per day.

[4] According to the mode of preparation both components of the synthetic racemic compound had the same isotope concentration. The total *l* isomer of the dietary phenylalanine therefore contained less deuterium than the *d* isomer. While it is probable that some of the latter was converted to tyrosine, since *d*-phenylalanine is known to support growth (22), the ratio between the amounts of the *d* and *l* forms utilized, and therefore the exact deuterium content of the converted phenylalanine, cannot be evaluated.

[5] Casein contains 4.5 per cent of *l*-tyrosine (8), so that the animals of Group I received 67 mg., and those of Groups II and III, 100 mg. of *l*-tyrosine per rat per day from this source.

stituent, and it has been shown (13) that dietary tyrosine replaces protein-bound tyrosine. This process, occurring continuously during the course of the experiment, would tend to dilute the isotope content of the tissue tyrosine by displacing isotopic tyrosine originally incorporated therein.

(c) Non-isotopic phenylalanine displaced from the tissue proteins by the influx of dietary phenylalanine would probably be converted into tyrosine which would follow the same course and exert the same effect as the dietary tyrosine.

Although the influence of these factors cannot be exactly evaluated, we are convinced that the conversion of phenylalanine to tyrosine is considerably faster and more extensive than is indicated by the figures given in Table I.

Conversion of Phenylalanine to Tyrosine As an Automatic Process

The continuous formation of tyrosine from dietary phenylalanine by normal, non-growing rats receiving abundant supplies of tyrosine in their diet may suggest that this conversion is an essential step in the oxidative degradation of phenylalanine.

Recent experiments with isotopes, however, have established the existence of other continuous conversion reactions, and the present results might therefore be interpreted in a different way. It has been shown that various amino acids and fatty acids are continuously interconverted and introduced into proteins and fats despite an adequate supply of the end-products of these reactions in the diet. Stetten and Schoenheimer (23) have shown that in rats palmitic acid is converted into lower fatty acids, into palmitoleic acid, and into stearic acid, although all these substances were supplied in the butter fat of the stock diet. The newly formed acids are utilized as fat constituents and enter ester linkages. The carbon chain of ornithine is not only used for arginine formation (24) but also for the formation of proline and glutamic acid[6] by non-growing animals on a casein diet which contains all these amino acids in considerable quantities. The use of N^{15}, furthermore, has demonstrated the formation by animals in nitrogen equilibrium of a number of other amino acids which were also abundantly supplied in the food.

[6] Unpublished observations.

It is difficult to regard these interconversion reactions observed in normal animals merely as steps in metabolic degradation. They seem rather to represent automatic and non-interruptable biochemical processes, of synthesis as well as degradation, which are balanced by an unknown regulatory mechanism so that the total amount of the body material and its composition do not change. The conversion of phenylalanine into tyrosine is probably one of these automatic reactions in which a great number, and perhaps most, of the body constituents are involved.

SUMMARY

1. *dl*-Deutero phenylalanine was prepared by treating non-isotopic phenylalanine with 84 per cent deutero sulfuric acid at 50° for 8½ days. Degradation of the amino acid to deutero phenylacetic acid indicated that 96 per cent of the deuterium was present in the phenyl ring.

2. The amino acid was added to a casein-containing stock diet of growing and of adult rats. Samples of tyrosine were isolated from the proteins of these animals, and their deuterium content determined. The tyrosine samples isolated from the internal organs contained a concentration of deuterium, indicating that about 20 to 30 per cent of this tyrosine was derived from deutero phenylalanine. The samples obtained from the proteins of muscles and skin had a lower isotope content.

3. In a third experimental series, non-isotopic tyrosine in addition to the deutero phenylalanine was added to the stock diet of adult rats. Despite the abundance of tyrosine in the diet, about 13 per cent of the tyrosine of the internal organs was derived from deutero phenylalanine.

4. The experiments indicate that tyrosine is automatically formed from phenylalanine. Furthermore, the results in conjunction with earlier findings show that tyrosine is continuously liberated from protein linkage and replaced by tyrosine of dietary and metabolic origin.

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SOME FACTORS INFLUENCING THE OXIDATION OF ALANINE BY LIVER TISSUE

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Effect of Flavin-Deficient Diet

It has already been shown that there is a decrease in the flavin-adenine dinucleotide content of boiled extracts of liver tissue from rats on diets deficient in flavin (1). Groen and Schuyl (2) observed that a flavin-deficient diet caused a diminished O_2 uptake of rat liver tissue and Axelrod, Sober, and Elvehjem (3) reported a similar diminution of O_2 uptake with alanine as substrate. This result has been confirmed, and it has also been found that addition *in vitro* of flavin-adenine dinucleotide causes a greater increase of O_2 uptake with tissue from flavin-deficient animals than with that from controls, and in the former case restores the O_2 uptake almost to normal levels.

The animals were fed on the flavin-deficient diet described by Ochoa and Rossiter (1). Control animals received the same diet plus 50 γ of riboflavin per day. The O_2 uptake was measured by the usual Warburg manometric technique. The rats were killed by decapitation, the tissue minced with scissors and ground in an ice-cold mortar with 5 volumes of the cold medium (1 per cent KCl plus 0.05 M phosphate buffer, pH 7.3), 1 ml. of the suspension was pipetted into the bottle, and at the commencement of the experiment 0.2 ml. of a 4.5 per cent solution of *dl*-alanine was tipped in from the side bulb. KOH filter papers were in the center well. In all experiments the O_2 uptake was measured over the period of 60 to 120 minutes. By this time the residual respiration of the tissue (which at the outset was variable and seemed to depend on the state of nutrition of the animal) was low. In Table I the

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net O_2 uptake is given; *i.e.*, the O_2 uptake with alanine minus the residual O_2 uptake.

Liver tissue from animals on the flavin-deficient diet had a lower O_2 uptake than that from controls on the same diet plus 50 γ of riboflavin per day (Table I). Application of Fisher's *t* test to the difference of means of figures for animals on the de-

TABLE I

Effect of Flavin-Adenine Dinucleotide on O_2 Uptake

The net O_2 uptake (in c.mm. per gm. of fresh tissue per hour) of rat liver preparations after the addition of 0.2 ml. of 4.5 per cent *dl*-alanine, with and without the addition of 5 γ of flavin-adenine dinucleotide, was measured over a period of 60 to 120 minutes; temperature 38°.

	Without flavin-adenine dinucleotide	With flavin-adenine dinucleotide	Increase
Flavin-deficient diet	220	340	120
	50	560	510
	80	530	450
	10	160	150
	120	450	330
	250	340	90
Mean ($\pm \sigma$)	122 ± 95	397 ± 148	275 ± 180
Flavin-deficient diet + 50 γ riboflavin per day	250	330	80
	240	320	80
	460	570	110
	380	500	120
	310	410	100
	440	480	40
	240	260	20
Mean ($\pm \sigma$)	331 ± 98	410 ± 113	79 ± 37

ficient diet and for those on the deficient diet plus 50 γ of riboflavin per day gives $t = 3.88$. For $P = 0.01$, $t = 3.11$. These figures are obviously significant. If flavin-adenine dinucleotide[1] (5 γ) was added to the system, there was always an increase in O_2 uptake. This increase was larger in the case of animals on the deficient diet. There is less than one chance in 50 that this

[1] A sample of pure flavin-adenine dinucleotide kindly sent by Professor Warburg to Professor Peters.

increase is fortuitous ($t = 2.92$; for $P = 0.02$, $t = 2.72$). The flavin-adenine dinucleotide often caused a change in the residual O_2 uptake which always had to be determined when the increase in O_2 uptake due to alanine was measured in the presence of flavin-adenine dinucleotide.

The fact that there is a diminution in (a) O_2 uptake in the presence of alanine in tissue preparation and (b) flavin-adenine dinucleotide content of boiled extracts of livers of animals on a diet deficient in flavin indicates a diminution of amino acid oxidase activity under these conditions. That the addition of flavin-adenine dinucleotide restores the O_2 uptake to normal would seem to indicate that the protein portion of the system is not altered.

Effect of Thyroid Treatment

It is known that thyroid treatment causes an increase and thyroidectomy a decrease in the O_2 uptake of many surviving tissues. This is true for liver tissue, as is instanced by the experiments of Dresel (4), Gerard and McIntyre (5), Meyer, McTiernan, and Aub (6), Ebina (7), McEachern (8), and Victor and Anderson (9). Klein (10) reported that thyroid treatment caused an increase in O_2 uptake of rat liver preparations in the presence of amino acids and concluded that this treatment causes an increase in d-amino acid oxidase activity of liver tissue. In experiments on the effect of thyroid treatment on tissue respiration in progress when the above paper appeared this effect has been observed for dl-alanine. Further, Klein (11) found no increase in the flavin-adenine dinucleotide content of boiled liver extracts of such thyroid-treated animals. There remained the possibility that with excess flavin in the diet this increase might be observed. Experiments have shown, however, that thyroid treatment caused no change in the flavin-adenine dinucleotide of livers of animals receiving a basal diet, which contained adequate amounts of all vitamins necessary for normal growth and development, even when an additional supplement of riboflavin was given.

The rats were fed on the basal diet described by Peters and Rossiter (12). Each animal was given by mouth 0.6 gm. of desiccated thyroid and 50 γ of riboflavin per day for 7 to 10 days. Controls received the same diet without the desiccated thyroid. Net O_2 uptakes in the presence of 0.2 ml. of a 4.5 per cent solution

of *dl*-alanine were determined (Table II) as in the previous section, and the flavin-adenine dinucleotide (Table III) as previously described (1).   Thyroid treatment caused a significant ($t = 4.73$;

TABLE II

Effect of Thyroid on O_2 Uptake of Liver Preparations

The net O_2 uptake (in c.mm. per gm. of fresh tissue per hour) of rat liver preparations after the addition of 0.2 ml. of 4.5 per cent *dl*-alanine was measured over a period of 60 to 120 minutes; temperature 38°.

Basal diet	Basal diet + 0.6 gm. desiccated thyroid per day
390	480
300	340
170	520
140	470
260	520
230	460
Mean (± σ) 248 ± 91	465 ± 66

TABLE III

Flavin-Adenine Dinucleotide in Boiled Extracts of Rat Liver

The results are expressed in γ per gm. of fresh tissue.

Basal diet + 50 γ riboflavin	Basal diet + 50 γ riboflavin + 0.6 gm. desiccated thyroid per day
66	89
52	103
107	62
90	80
79	84
128	
110	
66	
Mean (± σ) 87 ± 26	84 ± 15

for $P = 0.01$, $t = 3.17$) increase in the O_2 uptake of liver preparations but not in the flavin-adenine dinucleotide content of boiled liver extracts.

DISCUSSION

The decrease in the oxidation of alanine in liver preparations from animals fed on diets deficient in flavin seems to be due to a

genuine deficiency in the *d*-amino acid oxidase flavoprotein of Warburg and Christian (13). That this deficiency can be made good by the addition of flavin-adenine dinucleotide *in vitro* indicates that the protein constituent of the enzyme is present in the tissue but that it is less active because of a lack of flavin-adenine dinucleotide, the decrease in which has previously been observed. The addition of the nucleotide causes a rapid building of the flavoprotein, as happens when it is added to the flavin-free protein isolated by Negelein and Brömel (14).

Klein (11) thinks that the increase in alanine oxidation after thyroid treatment is probably due to an increase in the protein of the *d*-amino acid oxidase. As has been shown previously (1), in animals receiving large amounts of riboflavin there is no increase in the liver flavin-adenine dinucleotide above a certain level. It would appear that a protein element in the tissues governs this level, especially as most of the flavin-adenine dinucleotide of liver tissue suspensions is not dialyzable (unpublished observations). One might therefore expect that an increase in this protein would give a corresponding increase in the dinucleotide, especially if the diet contains large amounts of riboflavin. As this is not the case, it may be that an increase in the protein component (unaccompanied by an increase in the flavin component) leads to a displacement to the right of the equilibrium

$$\text{Protein} + \text{flavin-adenine dinucleotide} \rightleftharpoons \text{flavoprotein}$$
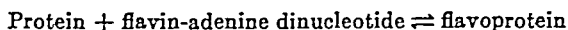
This is borne out by the increase in O_2 uptakes of partially purified enzyme preparations observed by Klein (11).

I am deeply grateful to Professor R. A. Peters for his continued interest and advice. Thanks are due to Dr. S. Ochoa for much help.

SUMMARY

1. In the presence of alanine the O_2 uptake of rat liver preparations from animals on a diet deficient in flavin is lower than that of similar preparations from animals on the same diet together with large doses of riboflavin.

2. Addition *in vitro* of flavin-adenine dinucleotide causes a greater increase in O_2 uptake of liver preparations from animals

on the flavin-deficient diet than those from animals receiving adequate amounts of riboflavin.

· 3. The observations of Klein have been confirmed that thyroid treatment causes an increase in the O_2 uptake of rat liver preparations in the presence of alanine, and that there is no change in the flavin-adenine dinucleotide content of boiled extracts of rat liver even if large amounts of riboflavin are given in the diet.

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PURIFICATION OF TOMATO BUSHY STUNT VIRUS BY DIFFERENTIAL CENTRIFUGATION

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Crystalline preparations of tomato bushy stunt virus have been obtained by Bawden and Pirie (1) by means of a chemical method involving heat treatment at 60° and repeated precipitation with ammonium sulfate. Although strictly chemical methods were first used successfully in the writer's laboratory for the isolation of viruses in purified form (2), it was later found that such methods caused a measurable loss of activity in the case of tobacco mosaic virus (3), about a 90 per cent loss of activity in the case of latent mosaic virus (4), and almost complete inactivation in the case of tobacco ringspot virus (5). Milder methods for the purification of viruses were sought, and as a result a purely physical method involving filtration and differential centrifugation was evolved (6), by means of which several viruses have been purified and obtained in an essentially unaltered condition (4–10). It appeared desirable, therefore, to purify tomato bushy stunt virus by means of the purely physical method of differential centrifugation and to compare the properties of such preparations with those of preparations obtained by chemical means.

It has been the custom in this laboratory to freeze the diseased plants used as the source of virus, in order to denature and render insoluble normal proteins of high molecular weight, which might otherwise contaminate viruses purified by differential centrifugation. However, Bawden and Pirie (1) have stated that bushy stunt virus differs from other viruses in that it is denatured and inactivated on freezing and thawing. Since no experimental data were presented, it appeared desirable to reexamine the question of the effect of freezing and thawing on this virus. Bawden and

Pirie have also suggested that, because the preparations of bushy stunt virus are fully crystalline, a stronger claim can be made for their purity than for the liquid crystalline preparations of other viruses such as tobacco mosaic virus.   However, they have shown further that bushy stunt virus, like tobacco mosaic virus, can be completely inactivated by treatment with nitrous acid or with ultraviolet light without impairing its ability to crystallize.   Since Smith (11), in studies on bushy stunt virus, showed in 1935 that heat treatment at 50–60° resulted in a large reduction in the number of lesions caused by infectious juice, it did not appear improbable that the heat treatment at 60°, as used by Bawden and Pirie, might result in inactivation.   If the bushy stunt virus should become partially inactivated during purification, owing to the heat or chemical treatment, the crystalline preparation of bushy stunt virus, like those of partially inactivated tobacco mosaic virus, might have a low specific activity.   In this case, the crystallinity of the preparation would provide no evidence for purity with respect to virus activity.   The question of the relationship between virus activity and the ability to assume a crystalline form, the effect on bushy stunt virus of freezing and thawing, and the comparative properties of bushy stunt virus purified by chemical means and by differential centrifugation are considered in the present paper.

EXPERIMENTAL

Frozen Versus Unfrozen Plants As Starting Material—It has been found in this laboratory that, in general, purified preparations of viruses may be frozen and thawed without a great loss of activity, provided protective materials such as extraneous proteins, nutrient broth, or in some instances even low concentrations of salts are present.   There is, however, considerable inactivation, the amount differing with the virus, when the freezing and thawing are carried out in the absence of such materials (5).   Bawden and Pirie (1) gave no data on experiments involving freezing and thawing, but merely stated that the process denatured and inactivated bushy stunt virus but had no effect on other viruses with which they had worked.   In view of this apparently rather unusual behavior and the fact that the freezing and thawing process is so important for the successful utilization of the differen-

tial centrifugation method, a study of the effect of freezing and thawing on bushy stunt virus was undertaken. Experiments pertinent to the question as to whether frozen diseased plants may be used successfully as starting material for the purification of the virus are described in the following paragraphs, and other more detailed data will be presented elsewhere.

Young tomato plants about 4 to 6 inches in height were inoculated with tomato bushy stunt virus by rubbing two or more leaves of each plant with a bandage gauze pad saturated with the juice of a badly diseased plant diluted with about 10 parts of water. 3 weeks later the diseased plants were cut, divided into two portions of 5 pounds each, and one portion was placed in a room held at $-12°$. The other portion was immediately put through a meat grinder, 3 per cent by weight of dipotassium phosphate in the form of an aqueous 50 per cent solution was added, and after thorough mixing the juice was pressed from the pulp. The 1250 cc. portion of juice which was obtained was rapidly filtered through a $\frac{1}{4}$ inch layer of celite (Hyflo super-cel) and then centrifuged at about 3000 R.P.M. on an angle centrifuge to remove a small amount of green pigment. 1200 cc. of the juice, which contained 1.80 mg. of total nitrogen per cc. and 0.77 mg. of protein nitrogen per cc., were centrifuged in small stainless steel tubes for $1\frac{1}{2}$ hours at about 30,000 R.P.M., corresponding to an average centrifugal field of about 60,000 g. The preparation of the plant material and all subsequent operations, including the high and low speed centrifugations, were carried out at 4°. Immediately following the centrifugation, the upper two-thirds of the supernatant fluid was found to contain 1.65 mg. of total nitrogen per cc. and 0.64 mg. of protein nitrogen per cc. and to be inactive when tested at a dilution of 1:10; hence practically all of the virus was contained in the 0.13 mg. of protein nitrogen per cc. which was sedimented. The supernatant fluid was decanted and the small solid pellets were well suspended in 200 cc. of 0.1 M borate buffer at pH 7 and centrifuged on an angle centrifuge at about 3000 R.P.M. for 30 minutes. The supernatant fluid was saved and the large amount of green-colored insoluble material was washed with 20 cc. of 0.1 M borate buffer. The material which was rendered insoluble by high speed centrifugation was discarded, and the wash liquid was added to the main portion and again centrifuged for $1\frac{1}{2}$

hours at 30,000 R.P.M. The cycle of alternate high and low speed centrifugation was repeated twice and each time the volume of buffer used to dissolve the pellets was approximately halved. The final preparation (No. 4, Table I) of 18 cc. of four times ultra-

TABLE I

Comparative Data on Different Preparations of Purified Bushy Stunt Virus

Prep-ara-tion No.	Type of plant material	Juice used	Type of treatment and solvent*	Purified preparation		
				Yield per cc. juice	P	Car-bohy-drate
		cc.		*mg.*	*per cent*	*per cent*
1	Frozen tomato	2900	4 cr. cy., borate	0.002		
2	" Datura stramonium	3800	4 " " " 1 pptn. salt	0.066	1.57 1.64	8.5 6.5
3	Unfrozen D. stramonium	240	5 cr. cy., borate	0.31	1.74	11.4
4	Unfrozen tomato	1200	4 " " " 2 additional cr. cy., borate	0.095	1.61 1.57	10.8 7.8
4a	Frozen tomato	1400	4 cr. cy., borate 2 additional cr. cy., borate	0.070	1.66 1.54	7.1 7.1
5	" "	2100	4 cr. cy., water, 1 pptn. salt, 1 additional cr. cy., water	0.026	1.52	9.8
5a	" "	5100	Heat to 60°, 3 pptns. salt, 2 cr. cy., water	0.011	1.54	7.8
6	" D. stra-monium	900	4 cr. cy., water	0.16	1.77	10.9
6a	" "	1150	Heat to 60°, 3 pptns. salt, 2 cr. cy., water	0.14	1.54	8.6
7	Frozen Solanum nodiflorum	700	5 cr. cy., phosphate	0.028	1.62	7.3

* "Cr. cy." indicates centrifugation at high speed followed by solution of the resulting pellets and centrifugation at low speed. The borate and phosphate buffers that were used were 0.1 M and at pH 7.

centrifuged virus contained 1.01 mg. of total nitrogen per cc., corresponding to 6.3 mg. of protein per cc. The yield was 114 mg. or 0.095 mg. per cc. of juice used as starting material. The preparation was found to contain 1.61 per cent phosphorus and 10.8 per cent carbohydrate estimated as glucose. Comparative data on this and other preparations are assembled in Table I.

After 3 days at $-12°$, the other 5 pound portion of bushy stunt-diseased tomato plants was put through a meat grinder, dipotassium phosphate added, and after thawing 1400 cc. of juice were pressed out and treated in exactly the same manner as that from the unfrozen plants. Before ultracentrifugation, the juice contained 1.47 mg. of total nitrogen per cc. and 0.32 mg. of protein nitrogen per cc., and after ultracentrifugation the upper two-thirds of the supernatant liquid contained 1.39 mg. of total nitrogen per cc. and 0.24 mg. of protein nitrogen per cc. Some idea of the amount of protein rendered insoluble by the freezing process may be gained from the fact that the amount of protein nitrogen in the juice from the plants which had been frozen was less than half that in the juice from the unfrozen plants. The freezing process afforded a further advantage in that but 0.08 mg. of protein nitrogen per cc. was sedimented from the juice from frozen plants, only about 60 per cent as much as was sedimented from the juice from unfrozen plants. The final yield was 102 mg. or 0.07 mg. per cc. of juice used as starting material, a yield of the same order of magnitude as that obtained from unfrozen plants. The preparation (No. 4a, Table I) contained 1.66 per cent phosphorus and 7.1 per cent carbohydrate. Since the preparation from frozen plants contained somewhat less carbohydrate than that from unfrozen plants, both were made up to 50 cc. each with 0.1 M borate buffer at pH 7 and ultracentrifuged. This was repeated once and on analysis the six times ultracentrifuged preparation from unfrozen plants contained 1.57 per cent phosphorus and 7.8 per cent carbohydrate, and that from frozen plants 1.54 per cent phosphorus and 7.1 per cent carbohydrate. Since the two additional ultracentrifugations did not further reduce the carbohydrate content of the virus from frozen plants and caused only a slight decrease in the phosphorus contents, values of 1.55 per cent and 7.1 per cent are considered to be good approximations of the phosphorus and carbohydrate contents, respectively, of the purified material. These values are slightly higher than those of 1.3 to 1.5 per cent and 5 to 6 per cent for phosphorus and carbohydrate, respectively, reported by Bawden and Pirie (1) for virus purified by chemical means.

The isolation of similar preparations from frozen and from unfrozen plants demonstrated that the physical integrity of the material was not destroyed by the freezing process. The specific

virus activity of the two preparations was next determined by the half leaf method (12) in order to ascertain whether or not the preparation from frozen plants might be less active, or completely inactive, as might be inferred from Bawden and Pirie's statement that the virus is inactivated by freezing and thawing. Solutions in 0.1 M phosphate buffer at pH 7 and containing 10^{-4} gm. of protein per cc. were prepared and used as inocula. One preparation was administered to the left halves of the leaves of half of the test plants and the other solution to the right halves of the same leaves. The latter solution was then administered to the left

TABLE II

Activity of Purified Bushy Stunt Virus Preparations Obtained from Frozen and from Unfrozen Diseased Tomato Plants

The figures represent the average number of lesions per half leaf obtained on inoculation of thirteen or more leaves of *Nicotiana glutinosa* with the designated material. All solutions tested were at pH 7 and contained 0.1 M phosphate buffer.

Preparation obtained from	Concentration, gm. protein per cc.			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Unfrozen plants, 4 centrifugations.............	43.5	24.8		
Frozen plants, 4 centrifugations...............	53.3	25.6		
Unfrozen plants, 6 centrifugations.............	29.0	9.5	2.9	0.1
Frozen plants, 6 centrifugations...............	30.7	7.5	3.7	0.1

halves of the leaves of the remaining half of the plants and the right leaves received the other solution. From the results of the tests, which are given in Table II, it may be seen not only that the virus obtained from frozen plants was active, but that it did not differ significantly in specific activity from the virus prepared from unfrozen plants. The activity of the untreated infectious juices from frozen and from unfrozen plants used as starting material for the two preparations was also compared by the half leaf method, and, as may be seen from the results which are presented in Table III, no significant difference was found. It may be concluded, therefore, that freezing and thawing diseased plants do not result in the inactivation of bushy stunt virus and, hence, that

frozen plants may be used as starting material in the purification of this virus. As indicated in the introduction and demonstrated by the experimental results, the freezing process removes much protein of high molecular weight which might otherwise contaminate the purified virus preparation.

Chemical Versus Physical Methods for Purification—In order to compare the properties of preparations purified by different methods, virus was purified by differential centrifugation and by a chemical method essentially that described by Bawden and Pirie (1) except that frozen plants were used as starting material. To frozen, macerated, bushy stunt-diseased tomato plants was added 3 per cent by weight of dipotassium phosphate and, after thawing,

TABLE III

Activity of Juices Pressed from Frozen and from Unfrozen Tomato Plants Diseased with Bushy Stunt Virus

The figures represent the average number of lesions per half leaf obtained on inoculation of thirteen or more leaves of *Nicotiana glutinosa* with the designated material. All solutions tested were at pH 7 and contained 0.1 M phosphate buffer.

Juice from	Dilution of filtered infectious juices		
	1:10	1:100	1:1000
Unfrozen plants....................	5.3	3.2	0.3
Frozen plants.....................	8.6	2.2	0.4

the juice was pressed out and filtered through celite. A 2100 cc. portion of the filtered juice was subjected to four successive centrifugation cycles, as described in the preceding section, except that water instead of borate buffer was used to dissolve the pellets. Since this particular preparation was definitely green-colored, 20 per cent by weight of ammonium sulfate was added and the precipitate was collected by centrifugation and suspended in water. Centrifugation of this suspension effected the removal of green-colored insoluble material. The supernatant liquid was subjected to ultracentrifugation and the pellets were dissolved in water. As will be shown later, such treatment with ammonium sulfate does not cause a loss of virus activity. The final preparation (No. 5, Table I) of 13 cc. contained 4.15 mg. of protein per cc. having 1.52

per cent phosphorus and 9.8 per cent carbohydrate. The yield of virus was 0.026 mg. per cc. of juice used as starting material.

Another portion of the same batch of frozen plants used for the preparation just described was macerated and, after thawing, the juice was pressed out. A 5100 cc. portion of the unfiltered juice was heated to 60° in a beaker on an asbestos pad over an open flame with continuous stirring and, after cooling, a green-colored coagulum was removed by centrifugation at low speed. To the clear brown-colored supernatant liquid was added 1 kilo of ammonium sulfate and the small precipitate which formed was removed by centrifugation and dissolved in 450 cc. of water. This solution was centrifuged at low speed to remove a small amount of insoluble material, and the protein was again precipitated by the addition of 20 per cent by weight of ammonium sulfate. The precipitate was collected, dissolved in water, and the precipitation with ammonium sulfate repeated. The precipitate was dissolved in 70 cc. of water and subjected to ultracentrifugation at 30,000 R.P.M. for $1\frac{1}{2}$ hours. The supernatant liquid contained 1.8 mg. of protein per cc.; hence, much inactive protein of low molecular weight persisted despite the heat treatment and three precipitations with ammonium sulfate. The pellets obtained on ultracentrifugation were suspended in 70 cc. of water, centrifuged at low speed to remove much insoluble brown-colored material, and the supernatant liquid again subjected to ultracentrifugation. The pellets were dissolved in 16 cc. of water to give a solution from which practically nothing was removed on low speed centrifugation and which contained 3.7 mg. of protein per cc. having 1.54 per cent phosphorus and 7.8 per cent carbohydrate. The yield of 59 mg. of protein (Preparation 5a, Table I) corresponds to 0.011 mg. per cc. of juice used as starting material. The virus activity of this material prepared by a chemical method involving treatment at 60° and repeated precipitation with ammonium sulfate was compared by means of the half leaf method with that of the material isolated by a physical method involving five centrifugation cycles. A solution containing 10^{-4} gm. of the chemically isolated material per cc. in 0.1 M phosphate buffer at pH 7 gave an average of 5.7 lesions per half leaf on forty *Nicotiana glutinosa*, L., leaves, whereas a similar solution of the material isolated by five successive ultracentrifugations gave an average of 22.7 lesions

per half leaf on the other halves of the same leaves. Similar results were obtained in subsequent tests; hence it appeared that the chemical method for purifying bushy stunt virus caused much inactivation of the virus.

The experiment just described was repeated, except that the juice from frozen macerated *Datura stramonium*, L., plants diseased with bushy stunt virus and to which had been added 3 per cent dipotassium phosphate was used as starting material. A 950 cc. portion of the juice was filtered through celite and then subjected to four successive centrifugation cycles to give a final preparation

TABLE IV

Activity of Purified Bushy Stunt Virus Preparations (Nos. 6 and 6a, Table I) Obtained by Means of Differential Centrifugation and by a Chemical Method from Frozen Diseased Datura stramonium Plants

Test No.	Preparation obtained by	Concentration, gm. protein per cc.	Lesions*
1	Differential centrifugation	10^{-4}	22.7
	Chemical treatment	10^{-4}	5.4
2	Differential centrifugation	10^{-4}	16.3
	Chemical treatment	5×10^{-4}	4.0
3	Differential centrifugation	10^{-4}	7.0
	Chemical treatment	10^{-3}	4.0
4	Differential centrifugation	10^{-4}	33.6
	Chemical treatment	10^{-3}	13.0

* The figures represent the average number of lesions per half leaf obtained on inoculation of twenty-two or more leaves of *Nicotiana glutinosa* with the designated preparation. All solutions tested were at pH 7 and contained 0.1 M phosphate buffer.

(No. 6, Table I) of 145 mg. of material containing 1.77 per cent phosphorus and 10.9 per cent carbohydrate. The yield from the *Datura stramonium* plants was 0.16 mg. per cc. of juice, a yield much higher than that obtained from tomato plants. A 1150 cc. portion of the same juice which was purified by chemical means likewise gave a high yield of 161 mg. of protein (0.14 mg. per cc. of juice) containing 1.54 per cent phosphorus and 8.6 per cent carbohydrate. The virus activity of the two preparations (Nos. 6 and 6a, Table I) was compared by the half leaf method and, as may be seen from the results which are presented in Table IV, the sample purified by physical means gave many more lesions than

the chemically prepared sample at the same concentration. It may also be seen that the sample prepared by centrifugation gave many more lesions when tested at only one-fifth the concentration of the sample prepared by chemical means and that about 2 times as many lesions were obtained even when the concentration of the chemically prepared sample was 10 times that of the sample prepared by physical means. In this experiment, therefore, the chemical method yielded a preparation possessing less than 10 per cent of the activity of a sample prepared from the same starting material by physical means. It is obvious that the chemical method used for purifying bushy stunt virus causes a large amount of inactivation.

Smith (11) demonstrated in 1935 that heating the infectious juice from bushy stunt-diseased tomato plants for 10 minutes at 60° caused a reduction in the lesion count from an average of about twenty-five per leaf to about six per leaf, and heating at 50° a reduction to about ten to fifteen per leaf. It seemed possible, therefore, that the heat treatment involved in the chemical method might be responsible for much of the inactivation. Although Bawden and Pirie (1) used the heat treatment in 1938, they reported no tests of the activity of the infectious juice before and after heating; hence, it appeared desirable to determine the activity of the juice after heat treatment at 60° as used in the chemical method. Since Lauffer and Price (13) found tobacco mosaic virus to be inactivated by heat more rapidly at alkaline than at acid reactions, tests were conducted at pH 5.5, the hydrogen ion concentration of the juice as pressed from the plant, and also at pH 6.5, the hydrogen ion concentration of the juice when pressed from macerated plants to which dipotassium phosphate has been added. The virus activities were determined by the half leaf method, and before application to the test plants the samples were diluted with 9 parts of 0.1 M phosphate buffer at pH 7. The heat treatment at pH 5.5 caused a reduction in the average number of lesions per half leaf from 13.8 to 0.4, and at pH 6.5 from 14.4 to 2.3. In another experiment, in which bushy stunt virus purified by differential centrifugation was used at a concentration of 10^{-4} gm. per cc. in 0.1 M phosphate, heating to 60° in a water bath followed by immediate cooling caused a reduction in the average number of lesions per half leaf from 58.2 to 12.1 at pH

5.5 and from 34.6 to 14.6 at pH 6.5. It is obvious that the heat treatment causes much inactivation of the virus at both hydrogen ion concentrations and that, if there is a significant difference, it is in favor of more rapid inactivation at pH 5.5 rather than at the more alkaline reaction. It has been found that practically no precipitate is formed when the juice from diseased tomato plants is heated to 50° and that heating to about 55° is necessary in order to cause a coagulum to form. In some instances the juice from diseased *Datura stramonium* plants has been heated to 60° without effecting denaturation and coagulation of normal proteins. Furthermore, it has been demonstrated by differential centrifugation that the heat-treated juice still contains an appreciable amount of protein of low molecular weight. The results obtained on heat treatment of the untreated infectious juices from bushy stunt-diseased plants are in accord with those reported by Smith in 1935 and indicate that the heat treatment as used by Bawden and Pirie in 1938 must have caused much inactivation of virus. It may be concluded that the freezing process is greatly superior to heat treatment for the removal of extraneous protein material from bushy stunt virus.

Certain viruses, such as tobacco ringspot virus (5), are inactivated by precipitation with ammonium sulfate, even when the precipitation is carried out at 4°; hence it appeared desirable to determine the effect of precipitation with ammonium sulfate on bushy stunt virus. A sample of bushy stunt virus purified by differential centrifugation was precipitated twice at 4° by the addition of 20 per cent by weight of ammonium sulfate. The virus activity of the twice precipitated sample was compared with that of the original by the half leaf method on *Nicotiana glutinosa* at two different concentrations. The untreated sample gave an average of 21.0 and 3.8 lesions per half leaf at 10^{-4} and 10^{-5} gm. per cc., respectively, and the twice precipitated sample 19.5 and 4.2 lesions per half leaf, respectively. The results demonstrate that bushy stunt virus may be precipitated by 20 per cent ammonium sulfate at 4° without altering the virus activity appreciably. This fact is of some importance for, as mentioned previously, bushy stunt virus preparations purified by differential centrifugation occasionally retain a green pigment rather tenaciously. This green-colored material is rendered insoluble in

water by the ammonium sulfate treatment and may then be separated from the virus by centrifugation.   In another experiment, purified bushy stunt virus at a concentration of 3 mg. per cc. was allowed to stand in contact with 30 per cent ammonium sulfate for 8 days at 4°.   The solution was then diluted with 0.1 M phosphate buffer at pH 7 and tested for virus activity by the half leaf method against a control which had stood at 4° for 8 days and then been diluted with buffer containing the same amount of ammonium sulfate used in the test solution.   The virus preparation that had been subjected to 30 per cent ammonium sulfate gave an average of 18.2 lesions per half leaf on forty-five *Nicotiana glutinosa* leaves and the control solution gave an average of 16.2 lesions per half leaf.   In two similar experiments, one of which was carried out at 20°, the preparations subjected to 30 per cent ammonium sulfate gave slightly fewer lesions than the corresponding control solutions.

As a whole, the results indicate that the chemical method for the purification of bushy stunt virus causes much inactivation of the virus and that most, if not all, of this inactivation is due to the heat treatment at 60° and little, if any, to the precipitation with ammonium sulfate.   The nature of this inactivation is of some interest, for the protein is still soluble and, in accordance with the findings of Bawden and Pirie, the preparations yield good crystals. Photographs of crystals of a preparation (No. 3, Table I) obtained by differential centrifugation and possessing a high specific activity and of a partially inactivated preparation (No. 6a, Table I) obtained by chemical and heat treatment are reproduced in Fig. 1. In a preliminary experiment, an attempt was made to fractionate a partially inactivated preparation of bushy stunt virus by crystallization, but the specific virus activity of the first crop of crystals and of their mother liquor was about the same.   This result is in accordance with Bawden and Pirie's statement that the activity of a crystalline preparation is unaffected by recrystallizations and, if confirmed by experiments now in progress, would demonstrate quite conclusively that crystallinity is no criterion of purity with respect to virus activity.   In another crystallization experiment, in which a portion of Preparation 6a was used, approximately 50 per cent of the material was obtained in crystalline form, although the virus activity of this material was less than

10 per cent of that of a preparation obtained from the same starting material by differential centrifugation. The general situation appears to be analogous to that which prevails in the case of tobacco mosaic virus in which it is possible to inactivate partially a preparation or to mix active with inactive preparations and yet obtain the well defined needle-shaped crystals. It seems likely that the partially inactivated preparations consist of a mixture of fully active virus with fully inactivated virus whose properties, with the exception of the virus activity, are very similar to those of the active virus; yet until some means of separating the two are
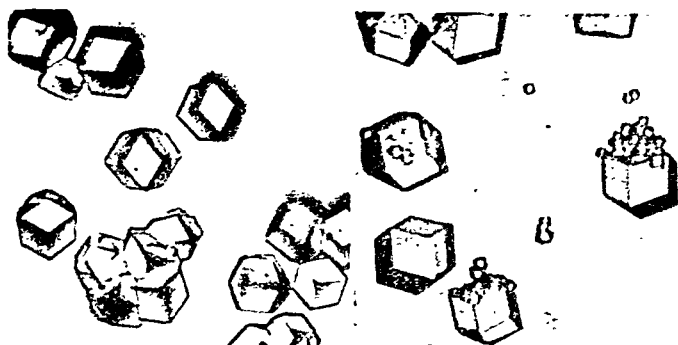
Fig. 1. Left-hand, crystalline material possessing a high specific activity and obtained from busy stunt virus Preparation 3. Right-hand, crystalline material possessing a low specific activity and obtained from bushy stunt virus Preparation 6a. × 89.6. (Photographs by J. A. Carlile.)

found the possibility that ultimate virus units of lowered activity exist must remain. However, the present experiments, in which material of low specific activity has been obtained in the form of crystals that appear to be indistinguishable from those of material of high specific activity, indicate that crystallinity cannot be used as a criterion of purity with respect to virus activity.

Isolation of Nucleic Acid—Bawden and Pirie (1) reported the isolation of nucleic acid from chemically purified preparations of bushy stunt virus but gave no analyses for carbon, hydrogen, or nitrogen and found only 6 to 7 per cent phosphorus. Since the nucleic acid isolated from other viruses appears to be of the yeast nucleic acid type and contains about 9 per cent phosphorus (5, 14),

and since Bawden and Pirie consider the nucleic acid from bushy stunt virus to be similar, it seems likely that their bushy stunt virus nucleic acid preparations contained appreciable quantities of extraneous materials. It appeared desirable, therefore, to repeat the isolation of nucleic acid from bushy stunt virus. Accordingly, 170 mg. of bushy stunt virus purified by differential centrifugation were subjected to the action of 5 per cent sodium hydroxide for 2 hours at 4°. The material was worked up according to the procedure previously described (5) to give 133 mg. of a protein component which was found to contain 0.24 per cent phosphorus. The 17.6 mg. of nucleic acid which were obtained were found to contain 35.71 per cent carbon, 3.87 per cent hydrogen, 15.45 per cent nitrogen, and 9.07 per cent phosphorus. The color tests given by the purified material were similar to those given by yeast nucleic acid and by tobacco mosaic virus nucleic acid and are indicative of the presence of a pentose and the absence of a desoxypentose. The fact that only 62 per cent of the phosphorus in the starting material was isolated in the form of nucleic acid and the protein component still contained 0.24 per cent phosphorus after 2 hours hydrolysis with 5 per cent sodium hydroxide is an indication that the nucleic acid is bound somewhat more strongly than in tobacco mosaic virus, for under similar conditions 90 per cent of the phosphorus in the latter virus may be isolated in the form of nucleic acid (14).

General Properties of Purified Preparations—The original source of the virus used in the present experiments was a small portion of a bushy stunt-diseased tomato plant. The writer is indebted to Mr. F. C. Bawden for generously supplying this material. Most of the properties of the virus produced in this laboratory and described in the preceding sections are essentially the same as those reported by Bawden and Pirie (1). Furthermore, the ultraviolet light absorption spectrum of a sample of virus purified by differential centrifugation, which was kindly determined by Dr. G. I. Lavin, was found to be essentially the same as that previously reported (Fig. 2). In accordance with the findings of Bawden and Pirie, solutions of the virus exhibit no double refraction of flow and the pellets that are obtained when the virus is subjected to high speed centrifugation are isotropic. These and other results indicate that the virus particles are essentially spherical in shape.

Studies on the diffusion constant of centrifugally purified bushy stunt virus (Preparation 5, Table I) are reported in the accompanying paper by Neurath and Cooper (15) who, using the Lamm refractometric method, found $D_{20} = 1.15 \times 10^{-7}$. The symptoms produced on tomato, *Datura stramonium*, bean, and cow-pea also appear to be the same as those described by Smith (11). A tomato plant diseased with bushy stunt virus and a healthy tomato plant of the same age are shown in Fig. 3. As may be
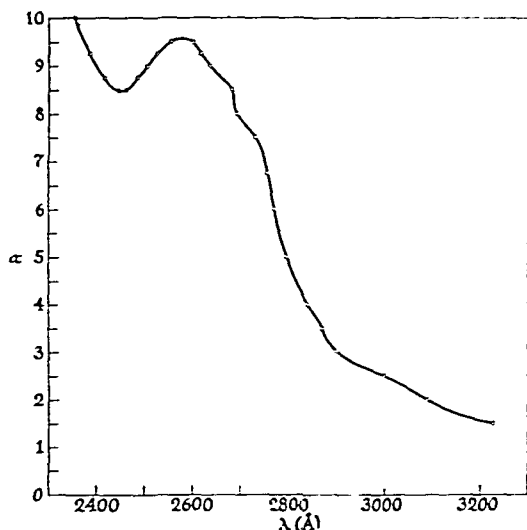
FIG. 2. Absorption spectrum curve obtained by Dr. G. I. Lavin for bushy stunt virus purified by differential centrifugation. The absorption coefficient, α, was calculated from the concentration expressed as mg. per ml.

seen from Table I, the phosphorus and carbohydrate contents of the virus preparations described in the present paper are slightly higher than the values reported by Bawden and Pirie (1). Although there appears to be no difference between the phosphorus and carbohydrate contents of the chemically and the centrifugally isolated preparations obtained from the same starting material (Preparations 5 and 5a, 6 and 6a, respectively, Table I), it would not be surprising if chemical treatment should cause a reduction in phosphorus and carbohydrate due to loss of nucleic acid, for such has been found to occur in the case of tobacco ringspot virus

(5)	However, the possibility of the existence of a real difference between the preparations described by Bawden and Pirie and those of the present investigation arose when the sedimentation constant of the latter was found to be about 132×10^{-13} (16), a value considerably lower than that of 146×10^{-13} reported by McFarlane and Kekwick (17) for the former preparations. At first it appeared that this difference might be due to some difference in the analytical centrifuge techniques of the two laboratories or to

FIG. 3. Left-hand, healthy tomato plant; right-hand, bushy stunt-diseased tomato plant of the same age. (Photograph by J. A. Carlile.)

a change caused by chemical treatment. As described in detail in the accompanying paper (16), the first of these possibilities has been eliminated through the generous cooperation of Dr. A. S. McFarlane who, using a sample of virus purified in this laboratory (Preparation 4a of Table I), was able to confirm the value obtained by Lauffer and the writer and others for the sedimentation constant of bushy stunt virus. The second possibility was rendered somewhat improbable when the sedimentation constant of preparations obtained by chemical means in this laboratory was not

found to differ essentially from that of virus purified by differential centrifugation (18). At present it appears that the difference in sedimentation constant between the chemically isolated preparations of Bawden and Pirie and the various preparations obtained in this laboratory may be due to some chemical treatment which has not been duplicated as yet in this laboratory, or possibly to an intrinsic change in the virus at some time between the completion of the work of Bawden and Pirie and the inauguration of the present investigation. The fact that, with the exception of the sedimentation constant, all other properties of the virus appear to be unchanged may be used as an argument against the latter possibility. However, the solution of the discrepancy with respect to sedimentation constant must be left to future work.

SUMMARY

Purified preparations of tomato bushy stunt virus have been obtained by differential centrifugation of the juices from frozen as well as unfrozen plants of tomato, *Datura stramonium*, and *Solanum nodiflorum* diseased with bushy stunt virus. The specific activity of virus from frozen plants was fully as great as that of virus from unfrozen plants, and there was no indication that the freezing and thawing of the diseased plants cause inactivation of the virus. In so far as determined, the properties of the purified preparations were the same regardless of the host plant. However, the yield of virus from *Datura stramonium* plants was 4 or 5 times that from tomato or *Solanum nodiflorum* plants. Purified preparations have also been obtained from similar starting materials by a chemical method similar to that described by Bawden and Pirie and involving heat treatment at 60° and repeated precipitation by means of ammonium sulfate. Although many of the properties of such preparations were apparently indistinguishable from those of preparations obtained by differential centrifugation, the specific virus activity of preparations obtained by chemical treatment was considerably lower. The inactivation caused by the chemical method appears to result from the heat treatment at 60° and not from precipitation with ammonium sulfate. The inactivation by the heat treatment is in accord with results published by Smith in 1935 and indicates that the purified preparations of bushy stunt virus obtained in 1938

Purification of Bushy Stunt Virus

by Bawden and Pirie by means of a method involving heat treatment at 60° must have consisted largely of inactivated virus.

Nucleic acid of the ribose type has been isolated from a preparation of bushy stunt virus purified by differential centrifugation. Bushy stunt virus appears to be a nucleoprotein which contains about 17 per cent of nucleic acid, has an ultraviolet light absorption maximum at about 2650 Å., a sedimentation constant of $S_{20w} = 132 \times 10^{-13}$, and a diffusion constant of $D_{20} = 1.15 \times 10^{-7}$. The sedimentation constant of preparations purified by chemical means was found to be the same as that of preparations obtained by differential centrifugation. The value is significantly lower than that of $S_{20w} = 146 \times 10^{-13}$ reported for virus purified by Bawden and Pirie. Purified preparations, whether isolated by differential centrifugation or by chemical means, may be obtained in the form of rhombic dodecahedric crystals having edges as great as 0.1 mm. Since preparations obtained by a chemical method involving heat treatment are partially inactivated, yet yield crystals similar to those of the fully active virus, crystallinity cannot be used as a criterion of purity with respect to virus activity.

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THE DIFFUSION CONSTANT OF TOMATO BUSHY STUNT VIRUS

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In 1938 Bawden and Pirie (1) isolated by chemical methods a crystalline protein from tomato plants infected with bushy stunt virus. For this material McFarlane and Kekwick (2) found, from sedimentation equilibrium measurements, a mean molecular weight of 7,600,000 and, from sedimentation velocity measurements, a value of 8,800,000, assuming the molecule to be spherical. The fair agreement between these two values was taken as an indication that the molecule does not depart considerably from a symmetrical shape.

An exact evaluation of the molecular weight and apparent molecular shape[1] of proteins from sedimentation velocity measurements requires that the diffusion constant be known. In the case of the tobacco mosaic virus, it was shown that the diffusion constant was much lower (4) and the molecular weight considerably higher than was expected on the assumption of spherical or moderately asymmetrical shape. The apparent molecular shape calculated from the diffusion and sedimentation constant agreed with the values obtained by other, independent methods (5).

In view of these findings it appeared of interest to determine the diffusion constant of the bushy stunt virus in order to obtain exact values for the molecular constants of this protein. A new method for its isolation by a purely physical process, involving differential centrifugation, and the chemical properties have been described by Stanley (6). The sedimentation constant of this material has been found to be 132.10^{-13} (7). The measurements

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[1] By "apparent molecular shape" we mean the shape of the molecules on the assumption of zero or negligible hydration (3).

reported in this paper were performed with the same material used in those experiments.[2]

Material and Methods

The virus was purified by four successive high and low speed centrifugations of the juice from diseased tomato plants. The material obtained in this way was found to have a higher specific activity than that obtained by chemical methods (6). The final solutions were slightly greenish and water-clear.

The material used for the diffusion experiments was dissolved in 0.1 M phosphate buffer of pH 7.1. Diffusion took place into a buffer solution of the same composition and pH. In view of the small amount of protein available it was necessary to dry the diffusion cell thoroughly before each run instead of following the customary procedure of rinsing it several times with the protein solution.

Diffusion measurements were carried out with the refractometric scale method of Lamm (8) by use of the apparatus described by Neurath and Saum (9) with minor modifications. The simple biconvex quartz lens was replaced by a two-element projector lens of 60 cm. focal length, with correction for spherical and chromatic aberration. A transparent 0.5 mm. scale of 50 mm. length was mounted inside the thermostat. A Westinghouse high pressure mercury arc lamp as light source allowed the time of exposure to be shortened from 90 seconds to 0.5 second, with an aperture of f:60 and Eastman Kodak spectroscopic plates, sensitized for the green. The photographic magnification factor was 1.1. All measurements were carried out at 25° ± 0.002°.

Results

In Table I values for the diffusion constant are listed as obtained from four independent experiments and for three different protein concentrations. The data refer to 25° and are not corrected for the viscosity of the solvent. Column 1 refers to the protein concentration, Column 2 to the time elapsed in seconds, Column 3 to the maximum height of the diffusion curve when the scale line

displacements are plotted against the positions of the displaced lines, and Column 4 to the area under the curves. Column 5 lists the photographic factor, $((l-b)/l)^2$, Column 6 the diffusion constants calculated by the maximum height method (Equation 1), and Column 7 the diffusion constants computed from Equation 2 (8). The equations used are as follows:

$$D = \frac{\mu^2}{2t}\left(\frac{l-b}{lG}\right)^2 \tag{1}$$

$$D = \frac{A^2}{(H_m)^2}\,\frac{1}{4\pi t}\left(\frac{l-b}{lG}\right)^2 \tag{2}$$

where D is the diffusion constant in sq. cm. per second, μ half the distance between the inflection points, G the photographic magnification factor, H_m the maximum ordinate, A the area under the curve, and t the time in seconds.

The agreement between the values for the diffusion constant is, if the data for the most concentrated solution is neglected, as good as can be expected under the conditions under which the experiments were performed. Exposure of the virus to room temperature over a period of several days is known to lead to partial inactivation and denaturation. The discrepancy between the values calculated from Equation 1 and those calculated from Equation 2 may possibly be taken as an indication of a certain degree of polydispersity. It is noteworthy, however, that the former are higher than the latter, whereas the opposite has been shown to be true in cases where the solutions were known to be polydisperse (10). If two or more molecular species with different diffusion constants were present at the beginning of the experiment,[3] the maximum concentration gradient (in the region of the original boundary) should be greater than in the case of a single component, owing to a superposition of the gradients produced by the individual diffusing components. This should lead to a lower average diffusion constant when calculated from the maximum height as compared with any method of calculation which takes into account the total area under the curve. The fact that in the present case the diffusion constant is higher when calculated from the maximum height may be taken to mean that in the region

[3] There is no indication of polydispersity by ultracentrifugation (7) or electrophoresis (11).

near the boundary diffusion proceeds in a normal way (and that only one molecular component is present), whereas in regions of low concentration gradients the diffusion rate becomes retarded. This would result in a more rapid falling off of the concentration gradient and consequently in a decrease in the total area under the

TABLE I

Results of Diffusion Measurements of Bushy Stunt Virus

H_m = maximum height of diffusion curves, A area under the curve, $((l-b)/l)^2$ the photographic factor, D_1 diffusion constant in sq.cm. per second, calculated from Equation 1, D_2 diffusion constant calculated from Equation 2.

Protein concentration (1)	Time (2)	H_m (3)	A (4)	$\left(\dfrac{l-b}{l}\right)^2$ (5)	D_1 (6)	D_2 (7)
per cent	*sec.*	*cm.* × 10¹	*sq.cm.* × 10²		10⁷	10⁷
0.8	141,300	350	593.3	0.8464	1.02	0.708
	222,300	255	581.6		1.02	0.814
	266,700	229	583.5		1.10	0.847
	322,500	207	595.8		1.19	0.920
0.4	60,900	313	464.5	0.8224	1.32	1.22
	89,400	252	460.5		1.40	1.26
	145,800	202	453.5		1.40	1.17
	171,300	186	462.5		1.42	1.22
0.4	201,600	168	452.9	0.8224	1.51	1.22
	247,500	154	439.4		1.38	1.17
	292,800	140	445.8		1.42	1.17
	333,900	134	454.1		1.50	1.16
0.2	92,400	140	242.0	0.8105	1.27	1.08
	116,520	130	252.6		1.29	1.08
	151,800	116	270.4		1.39	1.19
D^*, mean..					1.36	1.18

* The first four values omitted.

curve. Comparison of the experimental diffusion curve, plotted in normal coordinates, with the normal curve of error revealed indeed such a behavior.

The diffusion constants for the most concentrated solution are considerably lower than those obtained from the more dilute solutions. They also show a trend toward increasing with time. A decrease of the apparent diffusion constant with increasing con-

centration was also encountered with the tobacco mosaic virus (4) as well as with proteins of lower molecular weight (12), and is quite frequently due to mutual hindrance of the solute molecules from free diffusion. This concentration effect is much smaller here than in the case of the rod-shaped tobacco mosaic virus, as evidenced by the symmetrical shape of the diffusion curves even in the most concentrated solution investigated.

<div style="text-align:center">DISCUSSION</div>

The mean value for the diffusion constant of bushy stunt virus in dilute solutions is 1.27×10^{-7} (Table I) with a standard deviation of 0.13×10^{-7}. The standard deviation for the D_1 values alone is 0.09×10^{-13}. Correcting for the viscosity of the solvent, one obtains a value for D of 1.31×10^{-7} at $25°$ or 1.15×10^{-7} at $20°$. Taking the mean value of 132×10^{-13} for the sedimentation constant (7) and 0.739 for the partial specific volume at $25°$ (2), one obtains from the equation (13)

$$M = \frac{RTS}{D(1 - V\rho)} \tag{3}$$

a molecular weight of 10,600,000 with a standard deviation of 1,000,000. In Equation 3, M is the molecular weight, T the temperature, S the sedimentation constant, and D the diffusion constant, both for dilute solutions. V is the partial specific volume and ρ the density of the solvent.

The molar frictional coefficient, f_D, may be calculated from the diffusion constant according to the equation (14)

$$f_D = \frac{RT}{D} \tag{4}$$

and is in the present case 2.120×10^{17}. For a spherical, unhydrated molecule of the same volume it is (14)

$$f_0 = 6\pi\eta N \left(\frac{3MV}{4\pi N}\right)^{\frac{1}{3}} \tag{5}$$

where η is the viscosity of the solvent, N the Avogadro number, M the molecular weight, and V the partial specific volume. From this equation, f_0 is 1.672×10^{17}, and the dissymmetry constant, f_D/f_0, is 1.27. On the basis of Perrin's equation for the translatory

diffusion of ellipsoids of revolution (15, 3) one obtains a molecular axial ratio of 5.4:1 for a prolate ellipsoid and 5.8:1 for an oblate ellipsoid. It may be seen from these values that the apparent shape[1] of this protein departs from sphericity. The molecular axial ratio is about the same as that calculated for proteins of such widely different molecular weight as, for instance, hemoglobin and the heaviest component of hemocyanin of *Busycon* (3). A summary of the molecular constants is given in Table II.

Theoretically, the dissymmetry constant may also be greater than 1 if the solute molecules are hydrated to any significant extent. Kraemer (16) has derived an equation which, on the

<div align="center">

TABLE II

Molecular Constants of Bushy Stunt Virus from Sedimentation and Diffusion Data

</div>

S_{20} = sedimentation constant, D^{20} diffusion constant, M molecular weight, f_D molar frictional coefficient from diffusion, f_0 calculated molar frictional coefficient for spherical shape, f_D/f_0 dissymmetry constant, $(b/a)_p$ ratio of major to minor molecular axis for prolate ellipsoid of revolution, $(a/b)_0$ axial ratio for oblate ellipsoid of revolution, r gm. of solvent combined with 1 gm. of protein on the assumption of spherical shape.

S_{20}	D^{20}	M	f_D	f_0	$\dfrac{f_D}{f_0}$	$\left(\dfrac{b}{a}\right)_p$	$\left(\dfrac{a}{b}\right)_0$	r
10^{13}	10^7		10^{-17}	10^{-17}				
132	1.15	10,600,000	2.120	1.672	1.27	5.4	5.8	0.77

assumption of spherical shape, relates f_D/f_0 to the amount of hydration.

$$\frac{f_D}{f_0} = \left(\frac{rV_2 + V_1}{V_1}\right)^{\frac{1}{3}} \tag{6}$$

where V_1 and V_2 are the partial specific volumes of the solute and solvent respectively, and r the number of gm. of solvent combined with 1 gm. of solute. In the present case r is equal to 0.77.

The question whether $f_D/f_0 > 1$ is due to departure from spherical shape, hydration, or both cannot be decided without additional independent measurements of the dimensions of the protein molecules. In the case of a molecule of the size of bushy stunt virus hydration, to the extent of one or two water layers around the

surface of the molecule, should have only a negligible influence on the dissymetry constant. The increase in molecular radius due to this kind of hydration, amounting to about 2 Å., causes an insignificant decrease in the diffusion constant. However. x-ray data indicate that the hydration of this protein is not confined to the surface of the molecule, since successive wetting and drying of the crystals are accompanied by reversible swelling and shrinkage of the molecule as a whole (17). A detailed investigation of the problem of the shape of proteins of low molecular weight from the view-point of diffusion and viscosity has been under way in this laboratory and will be reported in a subsequent publication.

SUMMARY

The diffusion constant of ultracentrifugally prepared bushy stunt virus has been measured with the refractometric scale method. An average value of $D_{20} = 1.15 \times 10^{-7}$ has been obtained for dilute virus solutions.

From this value and the sedimentation constant, a molecular weight of 10,600,000 and a dissymmetry constant of $f_D/f_0 = 1.27$ has been obtained. Assuming negligible hydration, this would correspond to a molecular axial ratio of 5.4:1 and 5.8:1 for prolate and oblate ellipsoids of revolution respectively. About 77 per cent hydration would have to be assumed in order to account for the value of the dissymmetry constant on the assumption of spherical shape.

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STUDIES ON THE SEDIMENTATION RATE OF BUSHY STUNT VIRUS*

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Crystalline preparations of tomato bushy stunt virus, obtained by a chemical method from diseased tomato and *Datura stramonium* plants, have been described by Bawden and Pirie (2). More recently, this virus was purified by Stanley (14) by means of differential centrifugation of the infectious juices of these hosts and of diseased *Solanum nodiflorum* plants. During the course of the latter study it was found that heat treatment at 60°, as used in the chemical method of Bawden and Pirie, causes much inactivation of bushy stunt virus and yields preparations which, though crystalline, possess but a small percentage of the specific virus activity of preparations obtained by differential centrifugation. Studies conducted with the ultracentrifuge and reported in the first part of the present paper show that the sedimentation constant of centrifugally prepared bushy stunt virus is about 132 $\times$ 10^{-13} cm. per second in unit centrifugal field, a value considerably lower than that of 146 $\times$ 10^{-13} obtained by McFarlane and Kekwick (7) for a sample of the material isolated by chemical means by Bawden and Pirie.

Discrepancies in the sedimentation constants which have been reported for viruses are not unusual. Eriksson-Quensel and Svedberg (4) found the sedimentation constant of tobacco mosaic virus

corrected for water at 20° to be about 232×10^{-13} in the pH range 6 to 8, whereas Wyckoff (16) found this virus to have a sedimentation constant of 174×10^{-13} in a similar pH range. Price and Wyckoff (12) obtained a macromolecular substance having a sedimentation constant of 112×10^{-13} from plants diseased with tobacco necrosis virus, whereas Pirie, Smith, Spooner, and Mc-Clement (11) obtained two substances possessing comparable virus activity from plants infected with this virus, one with a sedimentation constant of 130×10^{-13} and the other with one of 58×10^{-13}. Pickels and Smadel (10) found a sedimentation constant of 49×10^{-11} for the elementary bodies of vaccinia, and Beard, Finkelstein, and Wyckoff (3) obtained a constant of 54×10^{-11} for this virus. Wyckoff (15) reported a value of about 245×10^{-13} for the sedimentation constant of the equine encephalomyelitis virus, and Sharp, Taylor, Beard, Finkelstein, and Beard (13) obtained a value of 273×10^{-13}. It is evident that in most of these cases the results from different laboratories are not in good agreement. Although in the case of tobacco mosaic virus the discrepancy can be explained as being due to different states of aggregation in the virus samples studied in the two laboratories, it was thought desirable to see to what extent the sedimentation rates on samples drawn from the same supply of virus could be duplicated by various workers. Bushy stunt virus was well adapted to this purpose, since it gives a sharp boundary in the ultracentrifuge, is reasonably stable, and does not appear to be susceptible to the aggregation process which affects tobacco mosaic virus. Samples of the same preparation of bushy stunt virus purified by differential centrifugation were sent to four laboratories, and the results obtained in all of these and in the authors' laboratory are brought together in the latter part of this paper.

EXPERIMENTAL

Materials and Methods

The virus samples used in this study were prepared by differential centrifugation from the juice extracted from previously frozen and thawed diseased tomato and *Datura stramonium* plants. They are described in detail in the preceding publication (14) and are referred to by the same numbers in the present publication. An air-driven ultracentrifuge of the type described

by Bauer and Pickels (1), equipped with a "turret" drive (9) and a Lamm scale optical system (5, 1), was used. It was operated at a speed of 18,000 R.P.M. at room temperature. The speed was measured stroboscopically with a General Radio Strobotac and was standardized against the vibrations of 60 cycle alternating current. The temperature of the rotor was measured by means of a thermocouple before and after each run, and the means were used in the computations of the corrected sedimentation rates. The following formula was used for this calculation

$$S_{20_w} = S_t \times \frac{\eta_t}{\eta_{20_w}} \times \frac{1 - V_{\rho_{20_w}}}{1 - V_{\rho_t}}$$

where S_{20_w} is the sedimentation rate that the material would have if it were moving under the influence of unit centrifugal field through a suitable solvent having the viscosity (η_{20_w}) and the density (ρ_{20_w}) of water at 20°. S_t is the measured sedimentation rate reduced to unit field, η_t is the viscosity of the medium, and ρ_t is the density of the solution, all at the temperature of the experiment. V is the partial specific volume of the solute, taken to be 0.739 (7). Three assumptions were made in order to carry out these calculations: (1) The viscosity of the solvent at a temperature t is equal to the relative viscosity of the buffer at 25° multiplied by the viscosity of water at the temperature t. (2) The density of the solvent at the temperature t is equal to the density of water at the temperature t multiplied by the relative density of the solvent measured at 25°. (3) The density of the solution is equal to the density of the solvent. For the case of very dilute virus solutions studied at temperatures differing only slightly from 25°, these assumptions should not be responsible for any appreciable error.

DISCUSSION

Five of the samples of bushy stunt virus described in the preceding paper (14) were studied in the ultracentrifuge. In the first experiment, virus from three of the samples was centrifuged at various concentrations in 0.1 M phosphate buffer[1] at about pH 7. The results are recorded in Table I. In the second column, the concentrations of the virus in mg. per ml. and, in the

[1] The molarity of the buffer refers to the phosphate radical.

third, the sedimentation rates times 10^{13} and corrected for water at $20°$ are recorded. A consideration of these data shows several facts of interest. The mean sedimentation rate is 131.6×10^{-13}, a value considerably smaller than that of 146×10^{-13} obtained by McFarlane and Kekwick (7) for chemically prepared bushy stunt virus. Most of the measurements recorded in Table I were made on a single preparation of virus, Preparation 2. It is at once evident that, even when the same sample of virus is centrifuged repeatedly, there is a considerable degree of variability

TABLE I

Sedimentation Rate of Centrifugally Prepared Bushy Stunt Virus in 0.1 M Phosphate Buffer at pH 7

Preparation No.	Concentration of virus	$S_{20_w} \times 10^{13}$
	mg. per ml.	
2	0.76	130.3
2	0.95	131.3
2	0.95	133.3
2	1.5	134.2
2	1.7	129.2
2	1.7	129.2
2	1.9	136.2
2	1.9	128.2
2	3.1	127.2
2	3.8	135.2
2	3.8	134.2
5	2.1	127.8
6	2.2	134.7
Mean value.................................		131.6

in the results obtained. The standard deviation of the distribution of sedimentation constants of Preparation 2 was calculated to be 2.9×10^{-13}, which would correspond to a probable error of $\pm 2.0 \times 10^{-13}$. Interpreted formally, this would mean that there is a 50 to 50 chance that a single determination of the sedimentation constant will differ from the mean and presumably actual value by no more than 2×10^{-13} unit. This degree of variability is not uncommonly large for studies on viruses, as is illustrated by the results obtained on bushy stunt virus by McFarlane and Kekwick (7) and by those presented in the following

section of this paper.   It is difficult to ascribe any definite reason for such variability.   Inspection of Table I reveals that there is no regular variation of appreciable magnitude of the sedimentation constant with the concentration of the virus,[2] so that this factor is probably not a determinant.

In the second experiment, in which only Preparation 2 of bushy stunt virus was used, the virus concentration was held constant at 1.7 mg. per ml., but the concentration of the phosphate

TABLE II

Sedimentation Rate of Centrifugally Isolated Bushy Stunt Virus (1.7 Mg. per Ml.) in Phosphate Buffers of Different Concentration

Molarity of phosphate buffer	$S_{20_w} \times 10^{13}$
0.00	125.8
0.00	126.2
0.00	127.2
0.00	131.1
0.01	133.2
0.01	133.2
0.01	136.2
0.03	134.2
0.05	133.2
0.10	129.2
0.10	129.2
0.30	125.8
0.30	132.4
0.30	134.0
0.50	124.2
0.50	126.2
Mean value......................	130.1

buffer was varied.   The results are presented in Table II.   Again the molarities refer to the phosphate radical, the buffer being composed of a mixture of monopotassium and dipotassium phosphate in a molar ratio of 1:2.2.   An inspection of the data in

[2] In a more detailed examination of this question (6), it was found that, on the average, an increase in bushy stunt virus concentration of 1 mg. per ml. causes a decrease of 0.25×10^{-13} unit in the sedimentation constant. However, this correlation does not meet the commonly accepted statistical standards for significance.

Table II will reveal a slight tendency for the sedimentation constant to be somewhat lower in solvents with no phosphate present and also in solvents with a phosphate concentration of 0.5 M. Stronger solutions could not be used, inasmuch as the virus is insoluble in molar phosphate.   In view of the variation in the magnitude of the sedimentation constant obtained for this same virus sample when the electrolyte concentration was constant (data of Table I), this apparent tendency is of doubtful significance.   In any event, the variation of the sedimentation constant with phosphate concentration is not great.   The mean value of the sedimentation constants reported in Table II is 130.1×10^{-13}, the standard deviation of the distribution of values is 3.7×10^{-13}, and the probable error is $\pm 2.5 \times 10^{-13}$.   By comparing these statistics with those calculated for the data of Table I, it becomes evident that the magnitude and the reproducibility of the sedimentation constant are not much affected by the introduction of phosphate concentration as a variable.

The difference between the sedimentation constants of the centrifugally purified and Bawden and Pirie's chemically purified bushy stunt virus is too great to be accounted for by random variations of the type just discussed.   The possibility that one isolates a somewhat different material by the two techniques must be considered.   However, the sedimentation constant of a sample (Preparation 6a of Table I (14)) prepared in this laboratory by means of a chemical procedure similar to that described by Bawden and Pirie was found to be 128.5×10^{-13} when the virus concentration was 7.5 mg. per ml. (6).   This value does not differ significantly from the sedimentation constant of centrifugally purified virus prepared from the same lot of starting material when determined under the same conditions (6).   It seemed possible, therefore, that the discrepancy between the value of 146×10^{-13} reported by McFarlane and Kekwick for a preparation obtained by Bawden and Pirie by chemical means and the average value of about 132×10^{-13} obtained in this laboratory for preparations obtained either by differential centrifugation or by chemical means was due to some systematic error arising during the sedimentation constant determinations in one or the other of the laboratories, rather than to a real difference between the sedimentation constants of the preparations.   The cooperative

experiments described in the next section were undertaken in order to investigate this point.

Reproducibility of Sedimentation Data from Laboratory to Laboratory

Samples of the same preparation of tomato bushy stunt virus (Preparation 4a of Table I (14)), which was isolated from diseased tomato plants by six successive centrifugation cycles with a 0.1 M borate buffer at pH 7 as the solvent, were studied in the laboratories of Dr. J. W. Beard of Duke University School of Medicine, Dr. A. S. McFarlane of the Lister Institute, London, Dr. A. Rothen of the Rockefeller Institute, New York, and Dr. J. W. Williams of the University of Wisconsin, and in our own laboratory. It may be noted that, at the time the determinations were made in each of the laboratories, no information other than the published work of McFarlane and Kekwick was made available and that in each case the determinations were of a routine nature and involved no special or extended study. The solution which was used in the various laboratories contained 2.5 mg. of the virus per ml. of a borax-HCl buffer, 0.1 M with respect to the borate radical and containing sufficient HCl to bring the acidity to about pH 7. The solvent had a relative viscosity at 25° of 1.02 and a relative density at 25.0° of 1.005. The sedimentations were effected with air-driven ultracentrifuges of the Bauer and Pickels type in three of the laboratories, with a motor-driven equilibrium centrifuge of the Svedberg type in one laboratory, and with an oil-driven ultracentrifuge of the Svedberg type in the remaining laboratory. Ultraviolet light absorption, schlieren, and scale methods were used to follow the boundaries. The results from all of the laboratories are brought together in Table III. The sedimentation constants presented in the last column were corrected to water at 20° by means of the formula described in a previous section, utilizing the data on the solvent just presented.

The results shown in Table III indicate that the degree of reproducibility from laboratory to laboratory is good. The mean values of the sedimentation constant for the various laboratories are 132.8, 136.3, 132.5, 130.1, and 134.0 $\times$ 10^{-13}. The extreme mean values differ by 4.6 per cent and the maximum deviation from the average of the four means is 2.4 per cent. The average of the four means, 133.1 $\times$ 10^{-13}, agrees well with the

average sedimentation constant reported in the preceding section for the virus in 0.1 M phosphate buffer at pH 7, 131.6×10^{-13}. The mean of all the values recorded in Table III is 132.8×10^{-13}, the standard deviation of the distribution of estimates from the various laboratories of the sedimentation constant is 3.5×10^{-13}, and the probable error is $\pm 2.3 \times 10^{-13}$. This indicates that the

TABLE III

Sedimentation Constants Obtained on Same Preparation (No. 4a) of Bushy Stunt Virus in Five Different Laboratories

In all cases the solution contained 2.5 mg. of virus per ml. in 0.1 M borate buffer at pH 7.

Laboratory	Type of centrifuge	Optical system	$S_{20_w} \times 10^{13}$
A	Air turbine	Scale	133.3
"	" "	"	132.3
B	" "	"	136.4
"	" "	Schlieren	136.4
"	" "	Absorption	136.0
C	" "	"	133.1
"	" "	"	131.1
"	" "	"	134.0
"	" "	"	131.9
D	Oil "	Scale	139.2
"	" "	"	134.2
"	" "	"	131.1
"	" "	"	132.1
"	" "	Schlieren	126.7
"	" "	"	131.9
"	" "	"	122.9
E	Motor-driven	Absorption	134.5
"	"	"	133.5
Mean value...			132.8

total variability in the results from all five laboratories is about the same as in the results from our own laboratory. These data leave no room for doubt that the sedimentation constant of centrifugally purified bushy stunt virus is actually near 132 or 133×10^{-13}. Furthermore, this study makes it seem highly probable that, when different sedimentation constants are obtained in two laboratories for what is presumed to be the same

virus, the difference is due to actual differences in the properties of the virus samples concerned and not to errors in centrifugation technique.

If 132×10^{-13} is taken to be the sedimentation constant of the bushy stunt virus, and if it is assumed that the virus particles in solution are spherical and not hydrated, one can calculate a molecular weight of 7.4×10^6 for this virus. This would correspond to a sphere with a diameter of about 26 mμ. If this sedimentation constant is used in conjunction with the diffusion constant of 1.15×10^{-7} sq.cm. per sec., obtained by Neurath and Cooper (8), one can calculate a molecular weight of 10.6×10^6.

SUMMARY

The sedimentation constant of centrifugally purified bushy stunt virus was found to be 131.6×10^{-13} for the virus dissolved in 0.1 M phosphate buffer at pH 7 and 132.8×10^{-13} for the virus dissolved in 0.1 M borate buffer at pH 7. Some variability was observed in a series of determinations of the sedimentation rate made with the same sample of virus, but no pronounced effect of changes in the concentration of phosphate buffer was demonstrated. In a study on a single preparation of bushy stunt virus in which five laboratories collaborated, it was demonstrated that the results from laboratory to laboratory are in good agreement. The sedimentation constant of about 132×10^{-13} which was obtained differs significantly from the value 146×10^{-13} reported previously for virus isolated by chemical means in another laboratory, and indicates that a real difference exists between the latter material and the samples used in the present investigation.

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THE PREPARATION AND PROPERTIES OF PITUITARY FOLLICLE-STIMULATING FRACTIONS MADE BY TRYPSIN DIGESTION*

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McShan and Meyer (1) have shown that the luteinizing activity contained in aqueous extracts of sheep pituitary powder is inactivated by treatment with trypsin, while the follicle-stimulating activity is relatively unaffected. This finding has been confirmed by Chen and van Dyke (2) and by Greep (3), although Abramowitz and Hisaw (4) reported that the follicle-stimulating activity of an extract, evidently prepared and purified by the method of Fevold (5), was inactivated. Chow, Greep, and van Dyke (6) reported recently that extracts prepared from fresh hog pituitary glands were inactivated after relatively long periods of digestion with crystalline trypsin, while a commercial trypsin preparation (Merck) did not destroy the follicle-stimulating activity.

The experiments described in this paper have as their object the development of a reproducible method by which a crude extract containing both follicle-stimulating and luteinizing activities can be converted to a product having follicle-stimulating activity only. The method includes procedures whereby toxic material and trypsin used in the digestion are removed. A preliminary report of this work has already been made (7); further developments are now presented along with a description of some of the physiological and chemical properties of the follicle-stimulating preparation obtained by this method.

Preparation

Acetone-dried sheep pituitary powder in 50 to 500 gm. quantities was extracted by shaking with distilled water in the proportion

of 1 liter of water to 100 gm. of powder. Three extractions were made in the earlier as compared to two in the later work. The latter abbreviated procedure is recommended because the solid content of the final product is reduced from 1.2 to 0.5 to 0.8 per cent without appreciable loss in activity.

The combined extracts were clarified by supercentrifugation. The gonadotropic activity was recovered by the addition of the combined extracts to 4 volumes of acetone followed by stirring. The precipitation was facilitated also by the addition of 3 or 4 drops of saturated sodium chloride solution for each liter of the aqueous acetone mixture. The precipitate was collected by centrifugation and suspended in water so that 1 cc. was equivalent to 250 mg. of original pituitary powder. Greater concentrations gave trouble in the subsequent heat precipitation of trypsin.

The aqueous extract was shaken 2 hours, supercentrifuged for 5 minutes, and treated at 37° for 3.5 hours at pH 8 with 40 mg. of trypsin[1] per gm. of original pituitary powder. The trypsin preparations should be low in diastase activity. The use of 20 mg. of trypsin was found to be almost as effective as 40 mg., and the follicle-stimulating activity was found previously to be little changed after digestion for 10 hours (1). The digest was centrifuged to remove a precipitate of inactive material. The aqueous digest was decanted into centrifuge tubes and then placed in a water bath and maintained at 75° for 20 minutes. The denatured and precipitated trypsin was removed immediately by centrifuging.

The completeness with which the trypsin was precipitated by the heat treatment was demonstrated by the failure of the heated solution to digest denatured hemoglobin when it was used as the substrate according to the method of Anson and Mirsky (8). The tyrosine content of the denatured hemoglobin digests was used as a measure of the tryptic activity of the gonadotropic digests before and after the heat treatment. Control solutions were made up of equivalent amounts of undigested pituitary extract, heat-inactivated trypsin, and denatured hemoglobin. The tyrosine was determined colorimetrically by use of the Folin

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[1] The trypsin used was similar to Sample 390,120 prepared by Fairchild Brothers and Foster, New York, and trypsin 1:125 prepared by the Pfanstiehl Chemical Company, Waukegan, Illinois.

and Ciocalteu (9) tyrosine reagent. The data given in Table I
show that the tyrosine values were high before the heat treatment,
but after heating they fell below that of the controls. These
results demonstrate clearly that the trypsin was completely re-
moved from the gonadotropic hormone digests by the heat
treatment.

After the heat treatment the follicle-stimulating digests were
dialyzed in cellophane tubes against 0.1 M acetate buffer of pH 4.
Adjustment to pH 4 was carefully executed so that toxic material
would be precipitated completely. It is important that the
supernatant liquid be clear at pH 4, as this is an indication that
the toxic material is precipitated. When the precipitation was
incomplete in one case, the final product was redissolved in water

TABLE I

Removal of Trypsin from Follicle-Stimulating Digests by Heat Treatment
0.1 gm. equivalent of pituitary digests used for each test.

Extract No.	Tyrosine content of Hb digests		
	Extract before heating	Extract after heating	Control
	mg.	*mg.*	*mg.*
50	2.65	0.13	0.53
100	2.55	0.15	0.19
102	2.88	0.07	0.31
103	3.03	0.24	0.26

and again adjusted to pH 4 by dialysis against acetate buffer to
remove the remaining toxic material, but this resulted in some
loss of activity. This latter step is not necessary, however, when
the first precipitation is complete as indicated by a clear solution.

The final product was obtained in solid form by the addition
of the digest, which had been adjusted to pH 4, to 4 volumes of
95 per cent ethyl alcohol followed by stirring and the addition of
2 or 3 drops of 2 N HCl for each 100 gm. equivalent of pituitary
powder. An efficient recovery is made by this procedure, while
the use of more than 4 volumes of alcohol results in the precipita-
tion of pigment from the solution. The fine white precipitate was
collected by centrifuging and dried by washing with alcohol and
acetone. The dry material was prepared for injections by dis-

solving it in the desired volume of water. Solutions of the powder were sterilized by Seitz filtration without loss of activity. The follicle-stimulating preparation has been used also in experiments on further purification.

Certain of the digests of the gonadotropic extracts were treated with the Folin and Ciocalteu (9) tyrosine reagent to show that protein contained in the extracts was hydrolyzed by the trypsin. The amino nitrogen content of several preparations, which was determined by use of the Sørensen formol titration method, was used as an indication of the degree to which the preparations were digested.

Physiological Characteristics—The follicle-stimulating preparations were assayed by injecting 21 day-old female rats with a total dose of 0.5 gm. equivalent of pituitary powder which usually ranged from 2.5 to 4 mg. in terms of dry material. The total amount of material for each rat was dissolved in 4.5 cc. of water and given in nine injections of 0.5 cc. each over 4.5 days. Autopsy was performed during the morning of the 6th day. Forty preparations have been prepared by the use of trypsin and the preparations and their assays given in Table II were selected at random from this group.

The ovaries obtained from the rats which received the untreated crude aqueous extracts had an average weight of 115 mg. and contained many corpora lutea. Test animals which received follicle-stimulating preparations had ovaries which contained only follicles, none of which was cystic. Macroscopic examination at autopsy revealed that 80 per cent of the ovaries contained clear follicles, while in 20 per cent the follicles were cloudy. The ovaries containing the cloudy follicles were sectioned and microscopic examination seldom gave evidence of luteinization of the granulosa, but there was some hypertrophy of the theca interna. When the dose of certain of the follicle-stimulating preparations was increased to 1 gm. equivalent of pituitary powder, ovaries weighing as much as 100 mg. were obtained. These large ovaries usually contained follicles only, but a small number of them had a few corpora lutea.

Greatly distended uteri were found in almost all cases when the ovaries were composed of clear follicles and weighed less than 60 mg. The uteri were less distended when the follicles were cloudy. The vaginae were open in 75 per cent of those with

ovaries weighing less than 50 mg. and which contained clear follicles. Rats with closed vaginae usually had distended uteri.

A few follicle-stimulating preparations were made by digestion of the gonadotropic extracts with 10 mg. of Wilson's high test trypsin per gm. equivalent of original pituitary powder. The ovaries produced by the administration of these preparations usually weighed less than 50 mg. One of the preparations when

TABLE II

Assay of Follicle-Stimulating Hormone Preparations Made by Trypsin Method

Extract No.	Dose	No. of rats	Ovaries	
			Weight	Qualitative response
	mg. equivalents		*mg.*	
1 (100)* (Undigested)	500	10	115	Many corpora lutea
2 (50)	500	6	43	All follicles
3 (100)	500	7	40	" "
4 (100)	500	12	57	" "
5 (100)	500	6	69	" "
6 (100)	500	6	64	" "
7 (100)†	500	4	76	" "
7 (100)‡	500	9	63	" "
8 (410)	500	6	53	" "
9 (500)†	500	3	80	" "
9 (500)‡	500	9	83	" "
10 (500)†	500	3	73	" "
10 (500)‡	500	6	57	" "
10 (500)‡	1000	3	96	Cloudy follicles

* The figures in parentheses indicate the gm. of pituitary powder used in making the preparations.

† Extract from Seitz filter before precipitation with alcohol.

‡ Dried preparation after recovery with alcohol.

given to immature rats in a total dose of 0.5 gm. equivalent of pituitary powder produced ovaries which had an average weight of 46 mg. These ovaries contained clear follicles only. The uteri of these animals were greatly distended, while the vaginae were not open. The follicle-stimulating activity was destroyed, however, when more than 10 mg. of this high test trypsin were used per gm. equivalent of original pituitary powder.

The qualitative difference in the results obtained with the

follicle-stimulating preparations as regards the size of the ovaries, the kind of ovaries produced, and the presence of occasional corpora lutea may depend upon the degree to which the gonadotropic extracts were digested by the trypsin, or to the incomplete inactivation of the luteinizing activity. It is possible also that interaction of the injected hormone with that secreted by the pituitary of the test animal might influence the ovarian response. However, two follicle-stimulating preparations which produced large ovaries were digested a second time, after which they produced smaller ovaries that contained clear follicles only. This suggested also that the degree to which the extracts were digested was important with regard to the physiological response. In order to obtain further information in this respect the amino nitrogen content of several of the follicle-stimulating preparations was determined by the Sørensen formol titration method, and used as a measure of the degree of digestion. Two follicle-stimulating preparations which produced ovaries with clear follicles only and greatly distended uteri were digested to a greater extent, as indicated by a greater amino nitrogen content, than were two other preparations which produced ovaries that were greater in weight and contained cloudy follicles with the occasional appearance of a few corpora lutea. These results indicate that the kind of ovarian response obtained depends upon the degree to which the gonadotropic extracts are digested by the trypsin, and that different trypsin preparations vary in effectiveness in regard to the digestion of gonadotropic extracts of sheep pituitary powder. Although the experimental methods are different, these results are believed to confirm those reported recently by Chow, Greep, and van Dyke (6) in which the kind of physiological response was found to be dependent upon the degree to which their extracts of fresh hog pituitary glands were digested by trypsin.

Intraperitoneal injection of 0.5 gm. equivalent of one of the follicle-stimulating preparations gave an average ovarian weight of 33 mg. with clear follicles. This figure is significantly greater than the control average ovarian weight of 13 mg. and further indication of gonadotropic response was found in the distended uteri and opened vaginae.

The absence of lactogenic and thyrotropic activities was demonstrated by the results obtained in the following assays. Thus, a total dose of 3 gm. equivalent of pituitary powder administered

intramuscularly to pigeons twice daily for 4 days resulted in unproliferated crop glands of the normal average weight of 1.5 gm., while a lactogenic hormone preparation administered at a 2 mg. dose level produced highly proliferated glands weighing 6.57 gm. It is interesting to note that the activity of this lactogenic hormone preparation was destroyed by trypsin.

The follicle-stimulating preparations were assayed for thyrotropic activity. The chicks used in the tests were 5 and 9 days old at the time of autopsy. A total dose of 1 gm. equivalent of pituitary powder given once daily during 4 days did not increase the weight of the thyroid glands. An undigested extract, however, increased the average normal weight of the thyroid glands from 3 to 20 mg. These data show that digestion of the gonadotropic extracts with trypsin destroyed the thyrotropic activity, and that follicle-stimulating preparations obtained from the trypsin digests did not contain thyrotropic activity.

Chemical Characteristics—The follicle-stimulating preparations were very soluble in water, since 175 mg. which were equivalent to 18 gm. of pituitary powder could be dissolved in 1 cc. of water. This concentrated solution was stored at 2° for 5 months without any detectable loss in activity. Solutions of the hormone at pH 7 to 8 were stable at 75° for 30 minutes. The dry powder was heated in the dry chamber of the autoclave under 15 pounds steam pressure for 1.5 hours with no significant decrease in activity. The solid content of the preparations usually varied from 0.5 to 0.8 per cent, which was 5 to 8 mg. per gm. of original pituitary powder.

The carbohydrate content of the product was relatively high. A value of 18.7 per cent calculated as glucose was manifested by the reducing action of the hydrolysate of the extract (10). Three different preparations when analyzed by the carbazole method of Gurin and Hood (11) were found to contain 22, 24.21, and 24.6 per cent carbohydrate, expressed as glucose. It was not possible to separate the carbohydrate from the follicle-stimulating activity by certain fractionation procedures or by dialysis. It is significant that the activity of the preparations was destroyed by certain amylase preparations such as ptyalin from saliva (1) and takadiastase. The preparations do not contain pentose sugars, as indicated by the benzidine test of Tauber (12), or ketose sugars, as indicated by the resorcinol test. The Molisch test was positive.

Fraenkel-Conrat *et al.* (13) have shown that unfractionated, follicle-stimulating, and interstitial cell-stimulating hormone preparations of the pituitary gland are inactivated by reduction with cysteine. We have confirmed this for follicle-stimulating preparations prepared by the trypsin method. The preparations were inactivated by treatment with 6 or 40 times their weight of cysteine at room temperature and at pH 7.8 for 48 hours.

DISCUSSION

The nature of the substance that produces follicle stimulation may be considered from two standpoints as regards the digestion of aqueous extracts of sheep pituitary powder with trypsin. First, the hormone that stimulates follicles may be a substance that is not attacked by trypsin but other proteins which are present are digested and as a result they are more readily separated from the follicle-stimulating activity. Second, the follicle-stimulating activity may be dependent upon an integral part of a protein, and if the conditions for the digestion are properly regulated the trypsin digests the protein to the extent that other possible physiological activities such as the luteinizing activity are destroyed, while the groupings or linkages that cause the growth of follicles are left sufficiently intact for this activity to be manifested by the digests.

The fact that trypsin is known to hydrolyze certain proteins to proteoses, peptones, and peptides can be reconciled to the second as well as the first hypothesis. The increase in the color produced by use of the Folin and Ciocalteu (9) tyrosine reagent after the gonadotropic extracts were digested with trypsin shows that protein associated with the gonadotropic activity was hydrolyzed. The kind of ovarian response obtained appears also to depend upon the degree to which the protein contained in the gonadotropic extracts is digested. Many corpora lutea were produced by the undigested extracts, while the luteinizing activity was virtually destroyed during the digestion with the enzyme. The ovarian response was dependent, however, upon the degree to which the preparations were digested. This was indicated by the results of the amino nitrogen determinations, as the preparations which produced cloudy follicles with the occasional appearance of a few corpora lutea were not digested to as great a degree as were other preparations which produced clear

follicles only. Greatly distended uteri were obtained also with the latter preparations.

Abramowitz and Hisaw (4) have reported that their follicle-stimulating preparations are inactivated by digestion with crystalline trypsin, and Chow, Greep, and van Dyke (6) reported recently that extracts of fresh hog pituitary glands were inactivated after long periods of digestion with crystalline trypsin. It is possible also to destroy the follicle-stimulating activity by the digestion of extracts of sheep pituitary powder with certain highly active non-crystalline trypsin preparations, as inactivation occurred when relatively large amounts of Wilson's high test trypsin were allowed to act for a short period of time. This inactivation produced by the high test trypsin in a relatively short time was probably caused in part by the presence of an enzyme other than trypsin which is more effective in the disruption of linkages that are indispensable for follicle-stimulating activity, as inactivation by crystalline trypsin required a long period of time. The destruction of the follicle-stimulating activity by crystalline trypsin appears to depend upon the conditions of the digestion, while the inactivation produced by non-crystalline trypsin may depend upon the presence of other enzymes as well as upon the conditions of digestion. It is believed that the inactivation by crystalline trypsin (4, 6) and perhaps that obtained by use of the high test trypsin indicate that there is a limit to which the protein contained in a gonadotropic extract can be hydrolyzed without destroying the follicle-stimulating activity.

The high carbohydrate content of the follicle-stimulating preparations and the failure to separate the carbohydrate by certain fractionation procedures or by dialysis give further support to the view that the follicle-stimulating activity is associated with the carbohydrate. Reduction with cysteine, however, indicates further that factors other than the carbohydrate are necessary for activity, and an attractive hypothesis is that inactivation by cysteine is associated with reduction of a disulfide grouping. It is well known that disulfides are reduced under mild conditions by cysteine with the formation of sulfydryl compounds and simultaneous oxidation of the cysteine to cystine. If this is true for the follicle-stimulating substance, the disulfide linkage is necessary also for follicle-stimulating activity.

It is proposed in the light of the results discussed that the

follicle-stimulating action is dependent upon a carbohydrate-peptide-like complex which contains an indispensable disulfide linkage, and that this complex obtained by digestion of pituitary extracts with trypsin may be an integral part of a larger protein molecule. There are certain linkages in the peptide or protein part of the complex which are also necessary for the activity as indicated by the inactivation by crystalline trypsin and perhaps by the inactivation produced by the high test non-crystalline trypsin preparation. While it is recognized that the results point toward this interpretation as regards the follicle-stimulating hormone, it is offered only as a working hypothesis.

SUMMARY

A method is given for making follicle-stimulating preparations from aqueous extracts of sheep pituitary powder by digestion with trypsin. The trypsin is removed from the digested extracts by heat treatment, and further purification is effected by dialysis. Luteinizing as well as lactogenic and thyrotropic activities are destroyed.

The product was found to be rich in carbohydrate, stable at 100° in the dry form, but inactivated in solution by ptyalin, taka-diastase, and cysteine. The results are discussed in relation to the nature of the follicle-stimulating hormone.

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SYNTHESIS AND EXCRETION OF TRIGONELLINE*

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Since Ackermann (1) has demonstrated that ingested nicotinic acid is partly excreted by the dog as trigonelline, and since this substance has been isolated from normal human urine by Linneweh and Reinwein (2), it became obviously important to estimate its excretion under a variety of conditions. In our studies on the estimation of nicotinic acid derivatives in human urine (3) we were greatly handicapped by the general unavailability of pure trigonelline and were obliged to attempt to prepare it synthetically. Two types of method for such a synthesis are available, that of Hantzsch (4) and that of Winterstein and Weinhagen (5). In testing these methods we were able to modify and simplify them as follows:

Trigonelline Hydrochloride Method, Modified from Winterstein and Weinhagen (5)—10 gm. of nicotinic acid ($\frac{1}{12}$ mole) and 12 gm. of methyl iodide ($\frac{1}{12}$ mole) in the presence of 15 ml. of dry methyl alcohol were heated at 150° for 3 hours in a 300 ml. pressure bottle in an autoclave. The mass was then dissolved in water and treated with an excess of moist silver oxide.[1] The filtrate was evaporated with 7 ml. of concentrated hydrochloric acid ($\frac{1}{12}$ mole) to a moist crystalline mass. The trigonelline hydrochloride was crystallized from 300 ml. of hot 90 per cent alcohol after standing in the refrigerator overnight. The precipitate was washed with cold alcohol and ether. 9 gm. of trigonelline hydrochloride melting at 258–259° were obtained. Concentration

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[1] At this point the filtrate can be evaporated and the free trigonelline (m.p. 140°) obtained by crystallization from alcohol.

of the mother liquor yielded another 2 gm. of the pure product. The 11 gm. represent a 76 per cent yield.

$C_7H_8NO_2Cl$. Calculated. Cl 20.46, equivalent weight 173.5
                 Found.   " 20.35,   "   " · 174.5 (titration)

Trigonelline Acid Sulfate Method—There is no record in the literature of the previous isolation and characterization of this salt. Heiduschka and Brüchner (6) on evaporating trigonelline with dilute sulfuric acid apparently obtained a ditrigonelline sulfate, judging from the percentage of sulfur given as 8.61. They give no other data on their salt. The trigonelline acid sulfate
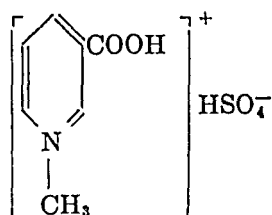
may be readily obtained by the following modification of the procedure used by Winterstein and Weinhagen (5). These authors did not isolate or describe the sulfate, but merely used it as an intermediate stage in the preparation of the hydrochloride. In a 1 liter, 3-neck flask, equipped with a mechanical stirrer, 24.6 gm. of nicotinic acid (0.2 mole) and 38.0 gm. of dimethyl sulfate (0.3 mole) were heated at 130° for 4 hours. This viscous mass was dissolved after cooling in 200 ml. of water and 1 ml. of 10 N sulfuric acid to decompose the methyl ester of trigonelline acid sulfate. This was evaporated to 60 ml. of a thick syrup, poured into 200 ml. of hot alcohol, and decolorized by boiling with Darco charcoal and filtering. After crystallization in the cold, 25.6 gm. of the pure trigonelline acid sulfate were filtered off and washed with cold alcohol and ether. Concentration of the mother liquor and addition of alcohol to 90 per cent gave a further crop of 4.7 gm. of pure crystals. The melting point of each sample was 199–200°. The yield was 30.3 gm. or 65 per cent of the calculated amount.

Trigonelline acid sulfate is easily soluble in water, 1 gm. dissolving readily in 1 ml. of water. At 25° about 2.1 gm. are soluble in 100 ml. of 90 per cent alcohol and only 1.2 gm. at 5°. Boiling

90 per cent alcohol dissolves about 8.5 gm. per 100 ml. In ether trigonelline acid sulfate is insoluble.

$C_7H_9NO_6S$. Calculated. S 13.6, equivalent weight 117.5
Found. " 13.56, " " 117.1 (titration)
" 13.51, " " 117.2

Hantzsch (2) found that on being heated with alkali trigonelline yielded methylamine. By distillation from strong alkali and titration of the distillate we have accounted for 96 to 98 per cent of the methylamine, identified in the form of N-methylbenzamide, m.p. 78–80°.

We have found that small amounts of trigonelline, when heated to 75° with 6 N KOH in the presence of a source of ammonia (ammonium salts or urea), yield a substance which gives a color identical with that of nicotinic acid in the Bandier and Hald (7) modification of the König reaction (cyanogen bromide and an amine). We believe that the ammonia closes the ring when methylamine is split out and gives nicotinic acid. On the basis of the color reaction, the conversion is about 70 per cent when one starts with less than 300 γ of trigonelline. Larger amounts of trigonelline decrease the yield progressively. We are attempting to adapt this procedure to a quantitative estimation of trigonelline in connection with the metabolism of nicotinic acid and its derivatives.

Our data thus far indicate, on the basis of the above reactions, that normal human subjects excrete daily only 1 to 3 mg. of nicotinic acid and derivatives (amide and glycine conjugate) compared with 30 to 50 mg. of trigonelline, and that nicotinic acid ingested in small doses (100 mg.) is excreted largely as trigonelline.

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AROMATIC SULFONIC ACIDS AS REAGENTS FOR AMINO ACIDS

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Precipitations of basic amino acids by aromatic sulfonic acids have repeatedly been reported. About 35 years ago, Suida described the salts of basic amino acids with two sulfonated dyes, orange II and crystal Ponceau (1). More recently, Kossel and Gross (2) recommended flavianic acid for the isolation and determination of arginine and other bases. Zimmermann described salts of quinizarinsulfonic acid with basic substances and with tyrosine and phenylalanine (3), and Ackermann (4) observed that 2,6-diiodophenol-4-sulfonic acid precipitates the basic amino acids.

The aromatic sulfonic acids are so strongly acidic that they may be expected to form salts with all types of amino acids. In the case of flavianic acid, Crosby and Kirk (5) verified this expectation by describing the microscopic characteristics of the crystalline flavianates of fourteen amino acids. It has apparently not been generally recognized, however, that many of the sulfonic acid salts of the neutral monoamino acids are sparingly soluble. Until the present, therefore, only the mono- and diflavianates of arginine, which at 0° have solubility products of 1.3×10^{-7} and 2.4×10^{-8} respectively, have come into general use (6). It has recently been reported (7), however, that naphthalene-β-sulfonic acid forms sparingly soluble salts with leucine and phenylalanine, as well as with arginine and histidine; and that flavianic acid forms sparingly soluble salts with tyrosine, phenylalanine,[1] and leucine. Conse-

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[1] Crosby and Kirk (5) stated that phenylalanine, glycine, and cystine failed to yield crystalline flavianates. In solutions acid to Congo red these salts were easily obtained.

quently, the solubilities of the amino acid salts of other aromatic sulfonic acids were investigated in the hope that new reagents suitable for the determination of amino acids might be found. The likelihood of finding such reagents has been greatly increased by the development of the solubility method. This technique does not require extremely insoluble salts, such as the arginine flavianates, but permits amino acid determinations to be made with the aid of salts the solubility product of which may be as high as 10^{-3} (corresponding to a solubility of about 0.03 mole per liter).

In Table I are reported the solubility products of salts of a number of aromatic sulfonic acids with various amino acids. Of the twenty-six sulfonic acids listed in Table I, twenty-five form sparingly soluble salts with phenylalanine, twenty-two with leucine, seventeen with histidine, fourteen with arginine, thirteen with tyrosine, and only three with lysine. It will be observed that the monoamino acids phenylalanine and leucine form sparingly soluble salts with more sulfonic acids than do the basic amino acids. Apparently the basicity of the amino acid involved is not the predominant factor governing the solubility of these salts. This conclusion is also supported by the fact that histidine is precipitated by many more sulfonic acids than is. the stronger base lysine. It can be seen, furthermore, that the solubilities of the corresponding leucine and isoleucine salts exhibit great differences. In all the cases reported in Table I the leucine salts are far less soluble. In fact, there are only a few isoleucine salts of sufficiently low solubility to warrant inclusion in the table.

The obvious fact that the solubilities of the amino acid salts vary greatly depending upon the structure of the sulfonic acid moiety need not be discussed in detail.

Several of the reagents listed in Table I merit further comment. Ackermann (4) reported that diiodophenolsulfonic acid (Reagent 23) does not form sparingly soluble salts with the monoamino acids, including proline. As may be seen in Table I, several monoamino acid salts have been prepared. These salts were obtained at 0°, however, while Ackermann apparently performed his experiments at room temperature.

In an earlier communication (7) it was reported that flavianic·acid (Reagent 25) forms difficultly soluble salts with several mono-

amino acids. The tyrosine salt is particularly insoluble and is therefore at present being employed in this laboratory for the determination of tyrosine in protein hydrolysates low in arginine.

The salts of 5-nitronaphthalene-1-sulfonic acid (Reagent 24) with glycine and with hydroxyproline are sufficiently insoluble to be employed in determinations of these amino acids. Experiments in this direction are in progress.

The leucine salts of 2-bromotoluene-5-sulfonic acid (Reagent 9) and 2-naphthol-7-sulfonic acid (Reagent 26) are less soluble than are the phenylalanine salts. As a consequence, these reagents have already been employed in this laboratory for the determination of leucine in protein hydrolysates.

2,5-Dibromobenzenesulfonic acid (Reagent 12) and 2,4,5-trichlorobenzenesulfonic acid (Reagent 13) promise to be helpful in the determination of phenylalanine in protein hydrolysates. The phenylalanine salts of these compounds are so much less soluble than are the leucine, arginine, and histidine salts that determinations of phenylalanine are possible even in the presence of an excess of these three amino acids.[2]

In conclusion, it may be mentioned that the utility of the aromatic sulfonic acids is by no means restricted to the determination of amino acids by the solubility method. These sulfonic acids should also prove helpful for the isolation and characterization of amino acids and organic bases in general.[3] An advantage attending the use of the aromatic sulfonic acids as reagents is that their amino acid salts may be isolated from acid solutions at pH 3 or below. It is not necessary, therefore, to adjust the reaction medium to neutrality, as it is in the case of picric and picrolonic acids. The sulfonic acids form sparingly soluble salts with several inorganic cations. Amino acid solutions investigated with the aid of the sulfonic acids should, therefore, be ash-free whenever possible.

[2] See the asterisk foot-note to Table I.

[3] Nitrobarbituric acid was recently recommended as a reagent for amino acids and organic bases by Redemann and Niemann (8).

TABLE I

Solubility Products of Amino Acid Salts of Aromatic Sulfonic Acids

The solubilities, unless otherwise indicated, were determined in N HCl at 0°. The salts of the sulfonic acids listed below with each of the following amino acids were investigated: l-alanine, l-arginine, l-aspartic acid, l-glutamic acid, glycine, l-histidine, l-hydroxyproline, l-isoleucine, l-leucine, l-lysine, dl-phenylalanine, l-proline, dl-serine, l-tyrosine, and l-valine. In most of the cases, the salts of l-cystine, l-cysteine, and dl-methionine were also investigated. Where no value is given, the solubility product of the salt in question was found to be greater than 4×10^{-2}. The values listed in bold-faced type are approximate solubility products, estimated in the manner described in the experimental section; the other values are accurately determined solubility products.

Reagent No.	Sulfonic acid	Employed as	dl-Phenylalanine $\times 10^{-4}$	l-Leucine $\times 10^{-4}$	l-Arginine* $\times 10^{-4}$	l-Histidine* $\times 10^{-4}$	l-Tyrosine $\times 10^{-4}$	Other amino acids $\times 10^{-4}$
1	p-Xylene-	Na salt	14	33			33	
2	p-Chlorobenzene-	"	120	334			51	dl-Methionine 100, l-cysteine 100
3	p-Bromobenzene-	"	35	120			190	" 100, " 50
4	p-Iodobenzene-	"	4.2	28				" 16, " 20
5	4-Chlorotoluene-2-	"	4.1	40			27	
6	2-Chlorotoluene-5-	"	32	16	**3.5**	**3.5**		
7	2-Chlorotoluene-4-	"	1.3	9.0	34	80		dl-Methionine 100
8	3-Chlorotoluene-	"	19	400				
9	2-Bromotoluene-5-	"	37	9.3				†
10	3,4-Dichlorobenzene-	"	4.8	26	0.0075	0.05		dl-Methionine 16
11	2,5-Dichlorobenzene-	NH4 salt	4.1	100		0.0019		" 50
12	2,5-Dibromobenzene-	Na salt	0.32	57	**0.25**	10	82	†
13	2,4,5-Trichlorobenzene-	Free acid	0.85	50	**0.16**	0.27	17	dl-Methionine 25
14	4-Nitrotoluene-2-	Na salt	20				28	Glycine 284
15	2,4-Dinitrobenzene-	"	55					
16	2,6-Dinitrotoluene-4-	"	50	67		**3.5**		

No.	Compound	Form						Amino acid salts
17	3-Nitro-6-chlorobenzene-	Free acid	16	81	3.5	3.5	49	dl-Methionine 50
18	3-Nitro-4-chlorobenzene-	"	20		0.5	0.5		
19	3-Nitro-4-bromobenzene-	"	19		0.02	0.02		
20	O-Benzyl-p-phenol-	"	1.3	3.5	0.0058	0.023		dl-Alanine 13, glycine 11, dl-serine 16
21	O-(2,4-Dinitrophenyl)-p-phenol-	"	0.29	1.2	0.011	0.00052	2.5	l-Lysine† 0.0023,* l-isoleucine 9, l-hydroxyproline 16
22	O-Tosyl-p-phenol-	"	0.11	0.28	0.056	0.01	1.7	l-Isoleucine 16, l-cysteine and l-valine 9, l-lysine 0.11,* l-cystine 0.058,* dl-methionine 0.81
23	2,6-Diiodophenol-4-	"	1.0	17	0.078	0.002	7.8	l-Hydroxyproline† 85, l-proline 68, dl-alanine 48
24	5-Nitronaphthalene-1-	"	0.67	131	0.016	0.61		Glycine† 5.2, l-hydroxyproline 8.7, l-lysine 0.25*
25	2,4-Dinitro-1-naphthol-7-(flavianic acid)	NH₄ salt	0.04‡	2.0	0.0013§	0.022‖	0.28	Glycine 25, l-lysine 0.013,* l-cystine 0.010*
		Na		5.7	0.00024¶	0.050**		
26	2-Naphthol-7-							†

* The arginine, histidine, lysine, and cystine salts, with the exceptions noted below, contain 2 moles of sulfonic acid per mole of amino acid. The solubility products of these ternary salts are, therefore, [sulfonic acid]² × [amino acid]. As a consequence, a comparison of the solubility product of a ternary salt with that of a binary salt is not a direct measure of their relative solubilities. For example, the arginine and phenylalanine salts of 2,5-dibromobenzenesulfonic acid (Reagent 12) have about the same solubility products. A saturated solution of the ternary arginine salt, however, contains about 0.02 mole of arginine per liter, whereas a saturated solution of the binary phenylalanine salt contains only 0.005 mole of phenylalanine per liter.

† The salts of l-cystine, l-cystine, and dl-methionine were not investigated in these cases.

‡ l-Phenylalanine flavianate.

§ Arginine monoflavianate; solubility determined in water.

‖ Histidine monoflavianate; solubility determined in water-methyl cellosolve, 4:1.

¶ Arginine diflavianate; solubility determined in 2 n HCl.

** Histidine diflavianate; solubility determined in n HCl.

EXPERIMENTAL

General Procedure—The ability of a given reagent to form sparingly soluble salts with amino acids was ascertained in the following manner: 0.5 cc. samples of a 0.4 N solution of the reagent in N HCl were added to 0.5 cc. samples of a 0.4 N solution of each of the amino acids in N HCl. The mixtures were kept at 0°, and in the event that any amino acid salt precipitated, known volumes of N HCl were progressively added to these mixtures until the precipitate redissolved. In this manner a rough estimate of the solubility of the salt was obtained. The solubilities found in this way are given in bold-faced type in Table I. A number of amino acid salts were prepared by dissolving the components in N HCl, isolating the salt at 0°, and recrystallizing it twice for elementary analysis of C, H, and N. The solubilities in N HCl, at 0°, of these salts were accurately determined, and are also recorded in Table I.

In order to conserve space, the analyses reported in Table II are limited to those amino acid salts that may prove useful for the isolation, purification, or determination of the amino acid in question.

The experimental account to follow is restricted to methods for preparing O-(2,4-dinitrophenyl)-*p*-phenolsulfonic acid and O-tosyl-*p*-phenolsulfonic acid, and modified directions for obtaining 5-nitronaphthalene-1-sulfonic acid. The other sulfonic acids listed in Table I were prepared according to procedures described in the literature.

O-(2,4-Dinitrophenyl)-p-Phenolsulfonic Acid[4]—The ammonium salt was used as reagent. It was prepared as follows: To a solution of 15 gm. of NaOH in a mixture of 150 cc. of water and 75 cc. of alcohol were added 68 gm. of sodium *p*-phenolsulfonate and 60 gm. of 2,4-dinitrochlorobenzene. The mixture was heated on a steam bath for 4 hours with frequent shaking. After cooling, 1.6 liters of water were added and enough HCl to make the solution acid to Congo red. The precipitate of dinitrophenol which formed was filtered off. Upon addition of 300 gm. of NaCl to the filtrate, the sodium salt of the dinitrophenyl ether of phenolsulfonic acid crystallized. Yield, 100 gm. It was successively transformed

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[4] Cook (9) described a compound of this structure obtained by sulfonation of 2,4-dinitrophenyl phenyl ether.

into the barium salt, the free acid, and the ammonium salt. 50 gm. of the ammonium salt were recrystallized once from 500 cc. of 95 per cent ethanol, and once from water before analysis. The air-dried salt was the monohydrate.

$C_{12}H_7O_4N_2S \cdot NH_4 \cdot H_2O$. Calculated. C 38.4, H 3.5, N 11.2, H_2O 4.8
375.2   Found.   " 38.6, " 3.4, " 11.05, " 4.9

The ammonium salt was also prepared by treating a hot solution of the sodium salt with a large excess of ammonium chloride.

O-Tosyl-p-Phenolsulfonic Acid—To a solution of 12 gm. of NaOH in 125 cc. of water and 65 cc. of alcohol there were added 55 gm. of sodium *p*-phenolsulfonate and 50 gm. of *p*-toluenesulfonyl chloride. The mixture was heated on the steam bath for $2\frac{1}{2}$ hours, cooled, and acidified with 3 N HCl. The sodium tosylphenolsulfonate crystallized out as big leaves. Yield, 135 gm. For analysis, the sodium salt was recrystallized from water. Dried over $CaCl_2$, the sodium salt contained 2 molecules of water of crystallization.

$C_{13}H_{11}O_6S_2Na \cdot 2H_2O$. Calculated. C 40.4, H 3.9, H_2O 9.3
386.2   Found.   " 40.4, " 4.2, " 8.9

A solution of the free tosylphenolsulfonic acid was obtained from the sodium salt over the barium salt. This solution was employed for the preparation of the amino acid salts.

5-Nitronaphthalene-1-Sulfonic Acid—100 gm. of α-nitronaphthalene were heated on a steam bath for 6 hours with 200 cc. of concentrated H_2SO_4. The mixture was cooled and poured into 2 liters of ice water. The dark suspension was filtered with the aid of charcoal, and 75 gm. of glycine were added to the filtrate. After standing at 0°, the glycine salt of 5-nitronaphthalene-1-sulfonate was obtained. It was recrystallized from water, and then transformed into the sodium salt by means of an excess of sodium acetate. Yield, about 25 gm. 20 gm. of the sodium salt were suspended in 400 cc. of 95 per cent ethanol, and water was slowly added to the boiling suspension until the sodium salt dissolved. The solution was treated with charcoal while hot and cooled to 0°; the faintly yellow sodium salt was filtered off and washed with 95 per cent ethanol and ether. The sodium salt was transformed into the free acid over the barium salt. The aqueous solution of the free acid was extracted three times with an equal

TABLE II

Analyses of Amino Acid Salts of Aromatic Sulfonic Acids

Unless otherwise indicated, the air-dried salt was analyzed. The results are given in per cent.

Sulfonic acid	Amino acid	Composition of salt	Mol. wt.	C		H		N		H₂O	
				Calculated	Found	Calculated	Found	Calculated	Found	Calculated	Found
2-Bromotoluene-5-	*l*-Leucine	$C_7H_7O_3SBr \cdot C_6H_{13}O_2N \cdot H_2O$	400.2	39.0	39.0	5.5	5.5	3.5	3.5	4.5	4.5
"	*dl*-Phenylalanine	$C_7H_7O_3SBr \cdot C_6H_{11}O_2N$	416.2	46.15	46.1	4.3	4.4	3.4	3.3	*	
"	*l*-Histidine	$(C_7H_7O_3SBr)_2 \cdot C_6H_9O_2N_3$	657.4	36.5	36.4	3.5	3.5	6.4	6.3		
3,4-Dichloroben-zene-	*l*-Histidine	$(C_6H_4O_3SCl_2)_2 \cdot C_6H_9O_2N_3$	609.4	35.5	35.4	2.8	2.9	6.9	6.75		
"	*l*-Arginine	$(C_6H_4O_3SCl_2)_2 \cdot C_6H_{14}O_2N_4$	628.4	34.4	34.4	3.5	3.5	8.9	8.8		
2,5-Dibromoben-zene-	*dl*-Phenylalanine	$C_6H_4O_3SBr_2 \cdot C_9H_{11}O_2N$	481.0	37.4	37.5	4.0	3.8	2.9	2.9	*	
2,4,5-Trichloro-benzene-	"	$C_6H_3O_3SCl_3 \cdot C_6H_{11}O_2N$	426.7	42.2	42.1	3.3	3.2	3.3	3.3		
O-Benzyl-*p*-phenol-	Glycine	$C_{13}H_{12}O_4S \cdot C_2H_5O_2N$	339.3	53.1	53.1	5.0	5.1	4.1	4.1		
"	*dl*-Alanine	$C_{13}H_{12}O_4S \cdot C_3H_7O_2N$	353.4	54.4	54.4	5.4	5.4	3.96	3.92		
"	*l*-Leucine	$C_{13}H_{12}O_4S \cdot C_6H_{13}O_2N$	395.5	57.7	57.6	6.4	6.4	3.5	3.6		
"	*dl*-Phenylalanine	$C_{13}H_{12}O_4S \cdot C_9H_{11}O_2N \cdot H_2O$	447.4	59.1	59.1	5.6	5.5	3.1	3.1	4.0*	4.0
"	*l*-Arginine	$(C_{13}H_{12}O_4S)_2 \cdot C_6H_{14}O_2N_4$	702.6	54.75	54.74	5.35	5.45	7.9	8.0	*	
"	*l*-Histidine	$(C_{13}H_{12}O_4S)_2 \cdot C_6H_9O_2N_3 \cdot \tfrac{3}{4}H_2O$	697.2	55.1	54.9	5.0	4.8	6.0	5.95	1.9	1.6
O-(2,4-Dinitro-phenyl)-*p*-phenol-	*l*-Leucine	$C_{12}H_8O_8N_2S \cdot C_6H_{13}O_2N \cdot H_2O$	489.3	44.2	44.1	4.7	4.6	8.6	8.7	3.7	3.8
"	*dl*-Phenylalanine	$C_{12}H_8O_8N_2S \cdot C_6H_{11}O_2N \cdot H_2O$	523.4	48.2	48.25	4.0	4.0	8.0	8.2	3.4	3.4

Compound	Amino acid	Formula	M.W.	%C calc.	%C found	%H calc.	%H found	%N calc.	%N found	%S calc.	%S found
O-(2,4-Dinitro-phenyl)-p-phenol-	l-Tyrosine	$C_{12}H_6O_5N_2S\cdot C_9H_{11}O_3N\cdot H_2O$	539.4	46.75	46.8	3.9	3.9	7.8	7.85	3.3	3.3
" "	l-Arginine	$(C_{12}H_6O_5N_2S)_2\cdot C_6H_{14}O_2N_4\cdot\tfrac{1}{2}H_2O$	808.1	41.5	41.5	3.6	3.6	12.0	12.75	1.0	1.1
" "	l-Lysine	$(C_{12}H_6O_5N_2S)_2\cdot C_6H_{14}O_2N_2\cdot 2H_2O$	802.0	41.8	41.7	3.9	3.9	9.6	9.6	4.2	4.1
O-Tosyl-p-phenol-	l-Leucine	$C_{13}H_{11}O_5S_2\cdot C_6H_{13}O_2N$	450.4	40.7	40.8	5.4	5.4	3.05	3.1	3.5	3.4
" "	dl-Phenylalanine	$C_{13}H_{11}O_5S_2\cdot C_9H_{11}O_2N\cdot H_2O$	511.5	51.7	51.5	4.9	5.15	2.74	2.74		
2,6-Diiodophenol-4-	dl-Phenylalanine	$C_6H_3O_4SI_2\cdot C_9H_{11}O_2N\cdot 2H_2O$	627.1	28.7	28.7	3.05	3.2	2.2	2.3	5.7	5.5
"	l-Tyrosine	$C_6H_3O_4SI_2\cdot C_9H_{11}O_3N$	607.1	29.05	29.5	2.5	2.6	2.3	2.3	3.4	3.2
"	l-Arginine	$(C_6H_3O_4SI_2)_2\cdot C_6H_{14}O_2N_4\cdot 2H_2O$	1062	20.4	20.4	2.5	2.35	5.27	5.24		
5-Nitronaphtha-lene-1-	Glycine	$C_{10}H_6O_5NS\cdot C_2H_5O_2N$	328.3	44.0	43.9	3.7	3.7	8.5	8.5		
" "	l-Leucine	$C_{10}H_7O_5NS\cdot C_6H_{13}O_2N$	384.4	50.0	50.2	5.3	5.3	7.3	7.3		
" "	l-Hydroxyproline	$C_{10}H_7O_5NS\cdot C_5H_9O_3N$	384.4	40.0	40.85	4.2	4.3	7.3	7.25		
" "	dl-Phenylalanine	$C_{10}H_7O_5NS\cdot C_9H_{11}O_2N$	418.3	54.55	54.6	4.3	4.3	6.7	6.7		
" "	l-Arginine	$(C_{10}H_6O_5NS)_2\cdot C_6H_{14}O_2N_4\cdot 2H_2O$	710.6	43.6	43.7	4.5	4.4	11.7	11.7	5.0	5.0
" "	l-Histidine	$(C_{10}H_6O_5NS)_2\cdot C_6H_9O_2N_3\cdot H_2O$	679.5	46.0	45.8	3.7	3.7	10.3	10.4	2.0	2.4
" "	l-Lysine	$(C_{10}H_6O_5NS)_2\cdot C_6H_{14}O_2N_2\cdot 3H_2O$	700.5	44.2	44.2	4.9	4.9	7.0	7.8	7.0	7.4
2,4-Dinitro-1-naphthol-7-	l-Leucine	$C_{10}H_5O_8N_2S\cdot C_6H_{13}O_2N\cdot 2H_2O$	481.4	39.9	40.1	4.8	4.8	8.7	8.7	7.5	7.5
" "	l-Phenylalanine	$C_{10}H_6O_8N_2S\cdot C_9H_{11}O_2N$	470.4	47.7	47.7	3.6	3.7	8.8	8.7		
" "	l-Tyrosine	$C_{10}H_6O_8N_2S\cdot C_9H_{11}O_3N\cdot H_2O$	513.4	44.4	44.5	3.7	3.8	8.2	8.1	3.5	3.5
2-Naphthol-7-	l-Leucine	$C_{10}H_8O_4S\cdot C_6H_{13}O_2N$	355.4	53.85	54.1	5.9	5.0	3.0	3.0	3.5	*

* These salts were dried over P_2O_5 in vacuo at room temperature before analysis.

volume of ether, the excess ether boiled off, and concentrated HCl added to induce crystallization. After recrystallization the free acid was obtained in long, pale yellow prisms. The air-dry acid was the dihydrate.

$C_{10}H_7O_5NS \cdot 2H_2O$. Calculated. C 41.55, H 3.8, H_2O 12.5
289.3   Found.   " 41.5, " 3.8, " 12.4

The fact that the nitronaphthalenesulfonic acid, isolated as the glycine salt, is the 5-nitro-1-sulfonic acid was ascertained by transforming its sodium salt into the acid chloride, m.p. 113–115°, and into the amide, m.p. 229–230°. The melting points reported in the literature are 113° for the chloride (10), and 229° for the amide (10).

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THE EFFECT OF ORGANIC COMPOUNDS UPON VITAMIN C SYNTHESIS IN THE RAT*

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A continued investigation of pure substances which accelerate vitamin C synthesis and excretion in rats (1, 2) has shown that several new series of compounds are as active as the terpene-like substances reported previously. Individual compounds in the new series, many of which are widely used clinically and in experimental work, are much more active than any of the compounds referred to in earlier reports. Although the very active substances differ greatly in chemical structure, they have an interesting common characteristic *in vivo*, functioning as nerve depressants. Among the most active compounds are four series of anesthetics (barbituric acid derivatives, polymeric aldehydes, sulfonemethanes, and halogenated aliphatic compounds) and two antipyretics, aminopyrine and antipyrine. Many of the bactericidal agents such as sulfapyridine, salicylates, and phenols are active to a much less degree. The more active compounds cause the animals to excrete from 10 to several hundred times more vitamin C than would otherwise be excreted on a given diet. In view of the fact that without any intake the quantities excreted each day are far in excess of the total body stores, the phenomenon obviously involves an increased rate of synthesis unless one postulates a normal very rapid turnover (synthesis and disposal) that might be blocked at the second step with resultant accumulation of the ascorbic acid.

There is evidence in the literature of a disturbance in vitamin C metabolism due to anesthesia. Zilva (3) found that when the vitamin was injected intravenously guinea pigs excreted more ascorbic acid during ether anesthesia than they did after similar injections when no anesthetic was used. The anesthetic did not prevent the fixation of the vitamin in the tissues, since higher amounts were found with anesthesia than without it. Zilva was not inclined, however, to regard his results as indicative of abnormal metabolism of the vitamin. Bowman and Muntwyler (4) observed a 10- to 15-fold increase in the urinary excretion of ascorbic acid following ether anesthesia in the dog; smaller increases were noted in both the rat and the guinea pig. The ascorbic acid content of the liver and kidney was found to be increased in the etherized rat (5); decreases were noted in rat adrenals (5, 6) and in several tissues of the guinea pig (5), and the serum ascorbic acid in the guinea pig was increased (7). In profound amytal narcosis, Lombroso and Cera (8) observed a decrease in the total ascorbic acid content of rat liver and a slight change in the ratio of dehydroascorbic acid to ascorbic acid.

The influence of narcosis on vitamin C economy has been studied by Bersin, Lauber, and Nafziger (9–12). These authors (9) measured the urinary excretion of ascorbic acid by rabbits after narcosis or local anesthesia and after various operations. They interpreted the decreased excretion values obtained by all procedures as indicative of an increased utilization (10). Bartlett, Jones, and Ryan (13) have also pointed out the increased need for vitamin C in surgical cases, but without specific reference to the possible effect of anesthetics. Wildbolz (14), however, could find no statistical justification for the opinion that ascorbic acid prevented or lessened the postoperative complications in which he was interested. In another study, Lauber et al. (15) found that narcotics generally produced a decrease in the vitamin C content of the adrenals and other "storage" organs.

The vitamin C content of the cerebrospinal fluid and brain was not affected by prolonged narcosis with opium derivatives or amylene hydrate in either humans or rabbits (16). Neither chloroform nor ether had a significant effect on the vitamin C content of the cerebrospinal fluid of apes or rabbits (17). A study of alcoholic patients has shown that a low vitamin C content in

the blood and cerebrospinal fluid is frequently observed without evidence of peripheral neuritis or psychosis, but, with the onset of the latter, there is a definitely subnormal content. Wortis et al. (18) believe that ascorbic acid may play a rôle in the metabolism of nerve tissue, and the work of Michetti et al. (19) contributes to this point of view. Melka (20) has shown that the ascorbic acid content of brain varies markedly in different sections, from 0.13 to 0.26 mg. per gm., compared to only 0.03 mg. per gm. in peripheral nerves. Dainow (21) believes that vitamin C exerts a definite control over the autonomic nervous system.

Evidence of the importance of vitamin C in detoxication processes has accumulated steadily in the literature. Papers from this and other laboratories have shown repeatedly that the tissue concentration of vitamin C is markedly lowered by some of the bacterial toxins and other toxic materials, and that there is a reduced tolerance for such substances by animals whose vitamin C reserves are depleted. Much of this literature has been referred to in recent reviews (22, 23), and additional investigations have pointed in the same direction. Thus the fatty degeneration of various organs after acute phosphorus poisoning was lessened in rabbits by treatment with vitamin C (24, 25); rabbits were also partially protected from phenol poisoning when vitamin C was administered (26); guinea pigs treated with 50 mg. of ascorbic acid for 8 to 10 days were made more resistant to lethal doses of phenylquinolinecarboxylic acid (27); chronic benzene poisoning was frequently accompanied by diminished urinary excretion of the vitamin (28) and a lowered blood concentration (29), and both man and experimental animals (30) appeared to be benefited by treatment with ascorbic acid (29, 31); in human lead poisoning the administration of ascorbic acid was followed by more rapid recoveries (32); in experimental animals (rats, guinea pigs, and rabbits) lead poisoning was alleviated by feeding apples (33), implicitly on the basis of providing vitamin C; and several investigators (34, 35) have reported a protective function of vitamin C against certain arsenicals.

In 1936, Daniels and Everson (36) reported a definite increase in ascorbic acid excretion when children (three) were given aspirin. Youmans and his colleagues (37) and van Eekelen (38) did not confirm this finding in healthy adults but Keith and Hickmans

(39) were able to produce a significant increase in vitamin C excretion in a large group of children with rheumatic fever by the administration of 50 grains of sodium salicylate and 100 grains of sodium bicarbonate.   In similar patients Richeri and Litter (40) found no influence on the blood level of ascorbic acid. Samuels, Ritz, and Poyet (41) confirmed our observation (2) of the increased excretion of ascorbic acid by rats when fed acetyl salicylate and extended their studies to include guinea pigs. The diuretics, salyrgan and neocinchophen, did not produce such an effect, though caffeine was slightly active.   The effect upon guinea pigs was only demonstrable when large dosages of ascorbic acid were given (33 mg. per 100 gm. of body weight).   Svirbely (42) reported tissue concentration changes with some of the above compounds, Einhauser (43) reported vitamin C to be effective in increasing the resistance of rats against lethal doses of barbiturates, and Haas (44) observed that the vitamin prevented the pyretic effect of 2,4-dinitrophenol.   Vedder and Rosenberg also observed a protective rôle for the vitamin in rats which were fed a peculiarly toxic jewfish oil (45).

The rôle of ascorbic acid as a detoxicating agent *in vivo* has been established from the types of evidence cited above, without a clear picture of the chemical basis for its action.   Other substances upon which the organism is known to rely for detoxication have been isolated frequently as conjugates of the toxic or foreign material.   References to conjugation with glucuronic acid, sulfates, glycine, cysteine, glutamine, ornithine, and methyl groups have been cited in reviews by Quick (46) and Young (47). It is generally accepted that such conjugation is for the purpose of either blocking active groups in or facilitating the removal of undesirable substances.   If ascorbic acid could be isolated from the urine of animals as a conjugated product with substances known to be toxic to the animal body following their administration, then direct and more satisfactory evidence would be available that ascorbic acid acts as a detoxifying agent.   Such evidence of conjugation has not yet been presented and experiments reported in this paper indicate that stable conjugation products do not occur in rat urine.   In our previous studies, however, it was shown that substances like the ionones, menthol, borneol, carvone, isophorone, piperitone, thujone, pulegone, and camphor, all of

which are known to be conjugated with glucuronic acid and often with sulfates, produce a simultaneous rise in the urinary excretion of ascorbic acid. The results provided by the earlier study and by the present data show a striking correlation between the metabolism of d-glucuronic acid and l-ascorbic acid. The relationship is all the more interesting because of the close analogy in their molecular structures.

EXPERIMENTAL AND DISCUSSION

A standardized technique, described in earlier publications (1, 2), was used in making the assays. Briefly, it consisted of adding definite amounts of pure substances each day to a basal diet of evaporated milk and feeding this mixture to albino rats maintained in individual metabolism cages. 24 hour samples of urine were collected in sufficient 12 per cent freshly prepared metaphosphoric acid, saturated with 8-hydroxyquinoline, to maintain a final concentration of not less than 3 per cent acid. In practice, 8 to 10 ml. of acid were found adequate for this purpose. The vitamin C content of these 24 hour urine specimens was determined by direct titration with 2,6-dichlorophenol indophenol and verified by a photoelectric colorimeter so that a correction could be made for other possible interfering agents (48, 49). Not less than nine 250 to 300 gm. standardized animals and usually ten or more were used to test each substance. The rats were raised from weaning on a stock ration (Dog Chow) until ready for the assays. On this ration they excrete about 2 mg. of vitamin C per day. A 2 day inanition period followed by 2 days on the milk diet alone was usually sufficient to reduce their excretion level to 0.2 mg. per day, at which value it remained constant on the milk diet alone. Contrary to a recent suggestion (50), we have recognized from the first that the composition of the diet *per se* is very important in relation to ascorbic acid excretion, but nominal variations in general composition do not exert changes that are comparable with those induced by specific stimulants. Furthermore, the response to specific stimulants is markedly dependent upon the basal diet used; hence our use of a standard assay diet of evaporated milk.

It will be noted that the substances listed in Table I are extremely active in causing an increased excretion of ascorbic acid.

<div align="center">Table I</div>

<div align="center">*Vitamin C Synthesis Induced in Rats*</div>

Daily ration, 35 ml. of evaporated milk + 20 mg. of test substance; nine or more animals for each test.

Test substance (barbiturates, hypnotics, and antipyretics)	Vitamin C excreted per 24 hr. period						
	1 day	2 days	3 days	4 days	5 days	6 days	7 days
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Evaporated milk only	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Barbital	0.4	0.9	1.4	4.0	6.7	7.9	8.6
Sodium phenobarbital	0.4	1.0	3.7	5.3	6.1	9.6	8.6
Phanodorn	0.6	1.2	2.7	4.1	3.4	4.2	
Evipal	0.5	1.2	1.4	1.6	1.8	1.4	1.8
Sodium amytal	0.3	0.2	0.4	0.6	0.8	1.0	1.3
Seconal	0.3	0.2	0.5	1.1	1.5	1.3	1.7
Nembutal	0.7	1.2	2.1	3.2	3.8	4.2	
Pentothal	1.4	2.6	3.0	3.9	4.2	3.8	4.2
Ipral	1.1	3.4	7.7	8.8	10.2	9.9	10.6
Alurate	0.7	1.2	1.8	2.1	2.0	1.7	2.4
Dial	0.3	0.4	0.3	1.0	2.0		
Chloral hydrate	0.6	0.7	0.7	0.8	0.7	0.8	
Paraldehyde	2.9	5.8	3.9	7.9	11.4	10.7	10.9
Chloretone	1.1	8.5	11.3	13.4	15.9	16.2	18.3
tert-Butanol (then changed to chloretone)	0.4	0.5	0.4	0.4	0.5	(To chloretone)	
Chloretone	3.4	8.0	11.5	14.6	16.8		
Bromural	0.5	1.2	2.2	2.6	2.1	1.3	
Tribromoethanol	0.3	0.2	0.3	0.5	0.5	0.6	
Trional	0.5	1.1	2.3	3.7	5.3	4.6	3.5
Urethane	1.0	0.4	0.4	0.8	0.7	0.4	0.7
Aminopyrine	0.9	1.8	3.0	4.2	5.9	7.4	
Antipyrine	0.4	2.3	5.4	6.1	6.5	4.7	
Dipyrone	0.5	0.8	1.6	2.6	1.6	1.7	
Acetanilide	0.4	0.4	1.0	1.1	1.2	1.2	
Phenacetin	0.3	0.4	0.4	0.4	0.4		
Aniline	0.3	0.3	0.4	0.5	0.6		
p-Aminophenol	0.2	0.4	0.7	1.0	0.8	0.7	0.8

The effect is interpreted as primarily the result of an increased rate of synthesis. All of the barbituric acid derivatives brought about this phenomenon and similar results were obtained with a

number of hypnotics and antipyretics. The most active compounds assayed were chloretone, paraldehyde, sodium phenobarbital, and calcium ipral. With these compounds the ascorbic acid excretion was regularly raised from the 0.2 mg. per day level on evaporated milk alone to 10 to 20 mg. The pyrazolone derivatives, aminopyrine and antipyrine, also exerted a marked stimulating action on ascorbic acid synthesis and excretion. From a structural point of view it was of interest to note that *tert*-butanol had very little effect compared with its trichloro substitution product.

The vitamin C content of urine from animals receiving 20 mg. of sodium phenobarbital daily was assayed biologically,[1] as described in the earlier paper. The biological data were in good agreement with the chemical data for determining the amount of vitamin C present. When an amount of urine equivalent to 0.5 mg. of ascorbic acid, determined by titration, was given as a daily supplement to the Sherman assay diet, seven guinea pigs, previously depleted for 12 days, gained an average of 10 gm. during a 21 day period. Their average scurvy score (on the basis of 0 to 24) was 5.3, and the average score for a control group of seven animals receiving a standard vitamin solution (0.5 mg. per day) was 4.7.

For the biological assay, urine with a high vitamin C content (20 to 25 mg. per day) was collected from the same animals over a period of 1 month. There was no evidence of injury to the animals during the test period. During a 2nd month the excretion level dropped to 12 to 20 mg. per day. A similar long continued excretion had been observed in earlier experiments with carvone and isophorone. When rats were continued for a long period on evaporated milk to which 20 mg. of chloretone were added daily, the urinary ascorbic acid levels continued to rise steadily to a maximum of 20 to 30 mg. per day, which was reached on the 12th to 14th day, and remained at that level for a 3 month test period. The high continued excretion level has been observed

for over 100 animals given chloretone for other test purposes. In several individual cases when urine was collected directly on a watch-glass and titrated immediately, values of 3.5 to 4.0 mg. of ascorbic acid per ml. were obtained, which, when calculated on the basis of average daily urine output, indicated a total 24 hour excretion of 50 to 60 mg. of the vitamin. Since the amount of ascorbic acid excreted per day through a long period was far in excess of the total quantity normally present in the animal, this finding considerably strengthens the probability of an increased rate of synthesis. It is also of interest that this concentration of ascorbic acid was much greater than had been found previously in any natural product.

The effects of a number of organic compounds of clinical and research interest are given in Table II. Several of these compounds are known to be detoxified by glucuronic acid (46, 47, 52). Sulfapyridine was only slightly active, but elicited a greater excretion than hydroxyethylapocupreine or sulfanilamide. The salicylates and phenols exerted little stimulation. Nicotinic acid and narcotine were fairly active. A biological assay of the vitamin C content of urine from animals receiving narcotine showed again that the chemical method gave essentially reliable results and that the values obtained were not due to other reducing substances. An amount of urine equivalent to 0.5 mg. of ascorbic acid produced an average weight gain of 50 gm. in six guinea pigs during a 21 day test period. The average scurvy score was 4.0. Alkaloids which were tested and found inactive or only slightly active (0.4 to 0.6 mg. of ascorbic acid per day) were papaverine, codeine, atropine, pilocarpine, cocaine, procaine, pantocaine, and intracaine. Caffeine, theobromine, and quinine were also inactive at a test level of 20 mg. per day. Among the alkaloid type of substances there was no apparent relation between nerve depressant effect and ascorbic acid excretion as noted for many of the other types of compounds.

In an earlier series of experiments the recovery of definite amounts of ingested ascorbic acid in the urine of rats was determined. The per cent of recoveries decreased rapidly as larger amounts of ascorbic acid were fed; with 100 mg. of ascorbic acid, very little more was recovered than when 50 mg. were fed. However, in more recent tests, intraperitoneal injections of 10

and 50 mg. of ascorbic acid dissolved in physiological salt solution permitted 60 per cent (range, 55 to 70 per cent) to be recovered during a 7 day test period with five animals. These values are similar to results obtained by Reiser (53). It might be argued from such data that not more than 60 per cent of the ascorbic

TABLE II

Vitamin C Synthesis in Rats Induced by Organic Compounds of Pharmacological Interest

Basal ration, 35 ml. of evaporated milk; nine or ten animals for each test.

Test substance	Vitamin C excreted per 24 hr. period						
	1 day	2 days	3 days	4 days	5 days	6 days	7 days
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Sulfanilamide, 50 mg.	0.2	0.3	0.5	0.6	0.4	0.5	0.5
Sulfapyridine, 50 "	0.3	0.4	0.7	0.9	1.2	1.1	1.3
Hydroxyethylapocupreine,* 50 mg.	0.2	0.2	0.3	0.2	0.4	0.5	0.4
Narcotine,† 20 mg.	1.4	3.6	4.1	4.7	5.5	5.3	5.2
Nicotinic acid, 50 mg.	0.9	1.1	1.3	0.6	2.2	3.6	3.6
Phenol, 25 mg.	0.7	0.6	0.5	1.1	1.3	1.0	1.0
Guiacol, 20 "	0.3	0.4	0.5	0.8	0.7		
Sodium diethyldithiocarbamate (20 mg.)	0.4	0.6	0.7	1.4	1.6		
Sodium salicylate, 20 mg.	0.7	1.0	0.9	1.3	1.2		
Phenyl " 20 "	0.3	0.3	0.4	0.8	1.0	1.1	1.4
Methyl " 20 "	0.3	0.3	0.4	0.4	0.4	0.5	

* Kindly supplied by Dr. L. H. Cretcher, Head of the Department of Pure Research, Mellon Institute.

† Alkaloids found to be essentially inactive, *i.e.* causing excretions less than 0.6 mg. per day, were codeine, atropine, papaverine, pilocarpine, cocaine, procaine, quinine, caffeine, and theobromine. The reported function of narcotine as an antiscorbutic agent *per se*, or as a supplement to *d*-glucuronic acid, has repeatedly been shown to be untrue (*cf.* Neuwiler (51)).

acid synthesized as a result of the ingestion of such compounds as reported here would be excreted in the urine, and hence that the excretion values represent minimum figures for the ascorbic acid actually synthesized rather than definite totals.

Attempts were made to isolate from the urines conjugation products of ascorbic acid with a number of the various compounds

listed above. These were all unsuccessful when procedures were followed of the type that lead to concentration or isolation of glucuronic acid conjugates. Neither was it possible to obtain an additional amount of reducing substance by acid hydrolysis of the urines. In further experiments to test for the presence of conjugated ascorbic acid, urine was collected directly from the animals onto a watch-glass and titrated immediately without acidification, with faint acidification with acetic acid (pH 6.5), and after hydrolysis for 10 minutes at 60° with acetic acid or hydrochloric acid at pH 3.5. In no case could additional ascorbic acid be obtained by acidification or hydrolysis. Neither could reducing material be extracted from cold acidified urine with ether, as should be true if the product were conjugated through other groups than those involved in the reducing action. Consequently, unless direct evidence becomes available to indicate conjugation, the ascorbic acid synthesized and excreted in rat urine can be regarded as unconjugated.

The properties of the vitamin contained in the rat urines with respect to precipitation by lead salts, solubility in organic solvents, and reaction with dinitrophenylhydrazine were identical with those of the free vitamin in solution. A discussion of the problem of separating the dinitrophenylhydrazine derivative from urine has been published by Roe and associates (54).

The present data considerably extend the number and types of molecular structures which produce accelerated ascorbic acid synthesis and they serve to emphasize further the improbability that these substances are direct precursors of the vitamin. Detailed studies of the synthesis by tissue slices will be recorded in a later paper (55). It is believed that the ascorbic acid is of endogenous origin, formed from tissue metabolites in much the same way as Lipschitz and Bueding (56) have demonstrated in the case of glucuronic acid.

The functional relationships of the active compounds cited in Table I point toward a close connection between vitamin C and the metabolism of nerve tissue; the increased synthesis of ascorbic acid constitutes a fairly rapid and continued response of the rat to at least an extensive number of nerve depressants. It is possible that the accelerated ascorbic acid synthesis is a protective mechanism available to the animal against foreign toxic substances

but there is no direct evidence that this is so. The phenomenon may also represent a disturbance of normal intermediate metabolism in which high ascorbic acid production is only one of a number of disturbed reactions.

SUMMARY

1. Ascorbic acid excretion by albino rats was greatly accelerated by a series of compounds that are widely used as nerve depressants in laboratory research and clinical practice.

2. All barbituric acid derivatives assayed were effective in stimulating ascorbic acid excretion. Sodium phenobarbital and calcium ipral were the most active; 20 mg. of each per day raised the average ascorbic acid excretion values from 0.2 to 10 mg. per day within a few days.

3. A group of chemically unrelated hypnotics had a similar effect; paraldehyde and chloretone in doses of 20 mg. per day caused average daily excretions of ascorbic acid of 11 and 18 mg. respectively.

4. Among the antipyretics which caused rats to excrete vitamin C, the pyrazolone derivatives, aminopyrine and antipyrine, were most effective; 20 mg. per day caused excretions of approximately 7 mg. of ascorbic acid within 5 to 6 days.

5. Phenols, salicylates, sulfanilamide, and sulfapyridine were only slightly active; 20 mg. of narcotine and nicotinic acid induced excretion of 4 and 5 mg. of ascorbic acid per day, respectively. Hydroxyethylapocupreine and a number of alkaloids caused practically no increased excretion. There was no evidence of correlation between nerve depressant action and causation of ascorbic acid excretion in this group of compounds.

6. Guinea pig assays for vitamin C in rat urines from animals receiving narcotine and sodium phenobarbital were conducted to verify the nature and amount of the reducing substance titrated with 2,6-dichlorophenol indophenol.

7. Rats continued to synthesize vitamin C for 3 months in

response to being fed 20 mg. daily of either sodium phenobarbital or chloretone, during which period they excreted 20 to 30 mg. of ascorbic acid per day. Individual animals excreted as high as 50 to 60 mg. per day (3.5 to 4.0 mg. of ascorbic acid per ml. of urine).

8. A much greater recovery of ascorbic acid was obtained in the urine of rats after intraperitoneal injection than resulted when the acid was fed.

9. No evidence was obtained to indicate that the urinary ascorbic acid was conjugated with any of the toxic substances fed, but its endogenous production appeared to be related to the animal's detoxication processes.

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THE EFFECT OF THE PITUITARY ADRENOCORTICO-TROPIC HORMONE AND OF VARIOUS ADRENAL CORTICAL PRINCIPLES ON INSULIN HYPO-GLYCEMIA AND LIVER GLYCOGEN

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In a recent publication we presented experimental evidence indicating that the anti-insulin[1] (glycotropic) effect of the anterior pituitary may be attributed to the adrenocorticotropic principle of that gland (1). We also reported that injection of desoxycorticosterone acetate, in contrast to corticosterone acetate, did not produce a significant anti-insulin effect under the same experimental conditions. In order to determine which of the different adrenal cortical principles exert a marked influence on carbohydrate metabolism and which do not, various steroids chemically related to corticosterone were studied with respect to their anti-insulin effect and their influence on liver glycogen. From the results of other investigators and our own data it appears that quantitative and also probably qualitative differences exist among the different adrenal cortical principles, with regard to their effect on carbohydrate metabolism.

EXPERIMENTAL

Anti-Insulin Test—The procedure followed in determining the anti-insulin effect of the various compounds in mice was the same as that previously employed by Jensen and Grattan (1).

The adrenocorticotropic preparations used in the various tests contained approximately one Moon unit per 5 to 10 mg. (2).

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[1] The term "anti-insulin" as employed by us refers only to the ability of a substance to counteract the hypoglycemia subsequent upon injection of insulin into an animal.

They were found to be free of lactogenic, thyrotropic, and gonadotropic effects at the dose levels employed in our experiments and were injected in aqueous solution at pH 7.5. The various steroid

TABLE I

Anti-Insulin Tests

The steroid compounds were administered in corn oil at the following concentrations: corticosterone and its acetate, desoxycorticosterone and its acetate, and progesterone, 0.5 mg. per 0.2 cc.; desoxycorticosterone and its acetate, progesterone, and methyltestosterone, 2.0 mg. per 0.2 cc.; methylandrostenediol, ethynylandrostenediol (suspension), and α-estradiol, 2.0 mg. per 0.3 cc.; 17-hydroxycorticosterone (suspension) and 17-hydroxy-11-dehydrocorticosterone (suspension), 0.5 mg. per 0.3 cc.; ethynyltestosterone (suspension) 1.0 mg. per 0.2 cc.

Preparation injected	Total amount per animal	Insulin dose per kilo	Total No. of animals	No. of convulsions	Per cent convulsions
	mg.	*units*			
Controls....................		1.5 or 2.0	202	179	89
Adrenocorticotropic*.............	5.0	1.5 " 2.0	128	21	16
Corticosterone acetate†..........	0.5	1.5	13	0	0
Corticosterone‡..................	0.5	2.0	30	3	10
17-Hydroxycorticosterone‡........	1.0	2.0	15	0	0
"	0.5	2.0	18	0	0
17-Hydroxy-11-dehydrocorticosterone‡........................	1.0	2.0	15	0	0
"	0.5	2.0	18	0	0
Desoxycorticosterone§............	2.0	1.5 or 2.0	26	20	77
" acetate§....	2.0	1.5 " 2.0	27	20	74
Progesterone§....................	2.0	1.5 " 2.0	41	34	83
α-Estradiol§......................	2.0	2.0	13	13	100
Methyltestosterone§..............	2.0	2.0	14	11	79
Ethynyltestosterone§.............	2.0	2.0	15	15	100
Methylandrostenediol§..........	2.0	2.0	13	12	92
Ethynylandrostenediol§	2.0	2.0	13	11	85

* The figures listed are the combined results obtained with various adrenocorticotropic fractions.

† Supplied by Dr. E. C. Kendall.

‡ Supplied by Dr. J. J. Pfiffner.

§ Supplied by Dr. E. Schwenk.

compounds were injected in a corn oil medium. Most of them were found to be readily soluble in a small amount of oil upon gentle heating on a water bath. Those failing to dissolve com-

pletely under these conditions were administered in a uniform suspension. The preparations were generally given subcutaneously in a single dose at the onset of the 6 hour fast. Whenever it was necessary to inject more than 0.2 cc. of an oil preparation,

TABLE II

Effect of Pituitary Adrenocorticotropic Hormone and of Various Adrenal Cortical Principles on Liver Glycogen

Preparation injected	Total amount per animal	Mode of administration		Total No. of animals	Average weight per animal at onset of fast	Average weight loss per animal during fast	Average liver weight per animal	Liver glycogen
	mg.				gm.	gm.	gm.	mg. per cent
Uninjected controls.......				72	19.9	0.90	1.18	616
Adrenocorticotropic.......	5.0	pH 7.5		28	20.3	0.23	1.32	2346
Corn oil controls..........		0.2	cc. oil	28	19.8	0.86	1.13	618
Desoxycorticosterone acetate....................	1.0	0.2	" "	28	20.8	0.86	1.11	606
Corticosterone*..........	0.5	0.2	" "	13	18.3	0.98	1.21	1368
" acetate†..	0.5	0.2	" "	28	19.7	1.07	1.19	2127
Corn oil controls..........		0.3	" "	12	20.8	0.73	1.16	302
17-Hydroxycorticosterone*.................	0.5	0.3	" "‡	24	21.3	1.31	1.43	3129
".................	0.25	0.15	" "‡	15	19.9	1.12	1.27	1927
Corn oil controls..........		0.8	" "	24	21.2	0.30	1.39	289
Desoxycorticosterone acetate§	2.0	0.8	" "	8	20.3	0.36	1.32	438

* Supplied by Dr. J. J. Pfiffner.
† Supplied by Dr. E. C. Kendall.
‡ Suspension.
§ Supplied by Dr. E. Schwenk.

one-half the dose was given at either side of the body. In all instances a control group of fasted animals receiving insulin only was included in the experiments. The anti-insulin response of the various compounds is recorded in Table I.

Effect on Liver Glycogen—Since the degree of resistance of an animal to insulin is apparently intimately connected with the amount of glycogen present in the liver, we have studied the influence of some of the preparations on the formation of liver glycogen. The experiments were carried out under conditions similar to those followed in the anti-insulin test. Male mice, weighing approximately 20 gm., were injected subcutaneously with the test preparation at the onset of the 6 hour fast. Glycogen determinations were generally conducted on four mice to a group. At the completion of the fasting period, during which time the mice had access to water, the animals were killed by the administration of 0.2 cc. of nembutal (1 cc. = 1 grain) and the livers removed immediately, immersed in distilled water, and dried with a soft cloth to remove all blood. Each group of four livers was then digested on a boiling water bath for $1\frac{1}{2}$ hours in 30 per cent potassium hydroxide (2 cc. per gm. of liver). 2 volumes of 95 per cent ethyl alcohol were next added and the mixture heated just to boiling. After standing overnight at 0° the digest was centrifuged, the precipitate dissolved in 10 cc. of warm water, and the glycogen reprecipitated with 2 volumes of 95 per cent ethyl alcohol. This second precipitate, after standing overnight at 0°, was removed by centrifugation, hydrolyzed with N sulfuric acid (1 cc. per gm. of liver) for 3 hours, and the solution then neutralized with sodium hydroxide, with phenol red as indicator. Duplicate glucose determinations were carried out with aliquot samples by the method of Shaffer and Hartmann, as modified by Somogyi (3).

When the amount of test material permitted, seven groups of four animals each were run on the various preparations. Control groups receiving no injection or an amount of corn oil comparable to that given the test groups were run at frequent intervals. As indicated in Table II, large doses of corn oil apparently lower the glycogen content of the liver. It is therefore advisable to administer the compounds in as small an amount of oil as possible. The effectiveness of the various preparations in promoting the deposition of liver glycogen is illustrated in Table II.

DISCUSSION

From the data presented in Tables I and II it is evident that the adrenocorticotropic factor of the anterior pituitary as well

as the adrenal cortical principles, corticosterone and 17-hydroxy-corticosterone, exert a pronounced anti-insulin effect and also markedly increase the deposition of liver glycogen under identical experimental conditions. It can also be seen that 17-hydroxy-11-dehydrocorticosterone produces a definite anti-insulin response, but unfortunately the amount of this compound available was insufficient to enable us to study its effect on liver glycogen. It is probable, however, that this cortical principle will also promote the deposition of liver glycogen.

On the other hand, the administration of desoxycorticosterone, at 4 times the dose level at which corticosterone, 17-hydroxycorticosterone, and 17-hydroxy-11-dehydrocorticosterone produce a definite anti-insulin effect and a marked increase in liver glycogen, failed to demonstrate any similar response. This observation is of importance, since it has generally been assumed that desoxycorticosterone is capable of completely alleviating the symptoms of adrenal insufficiency. It is unlikely that the difference in response is solely due to the rate of absorption. Sufficient desoxycorticosterone should have been absorbed at this comparatively high dose level to exert a definite influence on carbohydrate metabolism if the compound is at all active in this respect. Various other steroids tested for possible anti-insulin effect were also found to give a negative response.

It appears that those cortical principles which exhibit a positive anti-insulin response and produce an increase in the glycogen content of the liver will also favorably influence the work capacity of adrenalectomized rats, according to the observations of Ingle (4). Long and his associates have demonstrated that injection of corticosterone and of 11-dehydrocorticosterone into partially depancreatized rats causes an increase in glycosuria, while administration of desoxycorticosterone has little if any effect (5). Ingle recently observed that administration of 17-hydroxycorticosterone and of 17-hydroxy-11-dehydrocorticosterone will also augment the glycosuria of partially depancreatized rats (6). Recent clinical observations indicate that desoxycorticosterone acetate treatment has little or no effect in correcting the disturbance in carbohydrate metabolism which occurs in patients with Addison's disease (7). On the other hand Harrison and Harrison (8) have reported that the administration of desoxycorticosterone acetate prevents the

fall of the blood sugar in fasted adrenalectomized rats. It is of course possible that desoxycorticosterone may influence carbohydrate metabolism indirectly through its effect on the electrolyte balance. It may also be mentioned that Wells and Kendall (9) as well as Kuhlman *et al.* (10) have expressed the view that desoxycorticosterone is the only adrenal cortical principle capable of lowering the concentration of serum potassium, and that this factor is mainly involved in the regulation of the electrolyte balance.

These various observations seem to permit the conclusion that the different adrenal cortical principles do not affect carbohydrate metabolism, electrolyte balance, and life maintenance (adrenalectomized animals) to the same degree. Our findings and those of other investigators (11) indicate that only those adrenal cortical principles which contain either a keto or hydroxy group at C_{11} exert a significant influence on carbohydrate metabolism.[2] Whether derivatives of desoxycorticosterone containing either a keto or hydroxy group at positions other than C_{11} or C_{12} will exert an influence on carbohydrate metabolism has not yet been investigated. The function of the adrenal cortex may therefore be twofold, namely regulation (*a*) of the electrolyte balance and (*b*) of carbohydrate metabolism, these effects being elicited by different cortical principles.

The anti-insulin response produced by the adrenocorticotropic hormone and the corticosterone-like compounds (substitution in Ring III) is probably due to the ability of these substances to promote the formation of liver glycogen. We believe that the anti-insulin effect of the anterior pituitary can be attributed to the adrenocorticotropic principle and is mainly mediated through the adrenal cortex. Whether the anterior pituitary can also exert a direct and immediate influence on carbohydrate metabolism of the liver is still unsettled at present.

SUMMARY

The adrenal cortical principles, corticosterone, 17-hydroxycorticosterone, and 17-hydroxy-11-dehydrocorticosterone, exert a

[2] It has not as yet been definitely established whether these groups are at C_{11} or C_{12}.

pronounced anti-insulin effect and promote the deposition of liver glycogen.

Desoxycorticosterone and various other steroids exert little or no effect on insulin hypoglycemia and liver glycogen formation.

It seems that only those adrenal cortical principles substituted in Ring III (keto or hydroxy group) are principally involved in carbohydrate metabolism.

The adrenocorticotropic pituitary factor produces a definite anti-insulin (glycotropic) effect and also promotes the formation of liver glycogen.

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THE EFFECT OF TESTOSTERONE PROPIONATE ON INDUCED CREATINURIA IN RATS*

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That the gonads may be involved in creatine and creatinine metabolism was indicated when Rose (1) discovered a persistent creatinuria in children before puberty. After puberty the creatinuria ceases in boys, but continues to a lesser extent or in a cyclic manner in girls and women. In support of this finding, Read (2), McNeal (3), Remen (4), Bühler (5), Pizzolato and Beard (6), and others have claimed that castration in humans and animals leads to a creatinuria. In contrast, Tsun-Chee Shen (7), Kochakian and Murlin (8), Sandberg, Perla, and Holly (9), and others have not observed an induced creatinuria in men, dogs, or rats by castration. Considerable difference of opinion also exists concerning the effects of sex hormones upon creatine and creatinine excretion in hypogonadism and following castration. Bühler (10), Kun and Peczenik (11), Paschkis and Schwoner (12), and Kenyon *et al.* (13) find that androgens effect a decrease in hypogonad creatinuria. On the other hand, Pizzolato and Beard (6) report that castration in rats produces a creatinuria which is not decreased, but actually is increased by testosterone propionate administration.

In view of the many conflicting reports an investigation was undertaken on urinary excretion of creatine and creatinine in normal and castrated male rats kept on a creatine- and creatinine-free, high protein diet with and without testosterone propionate treatment and a repetition with a controlled intake of creatine.

EXPERIMENTAL

Equipment and Methods—The Dubos-Miller modification (14) of the Folin procedure (15) was slightly altered and adapted for

use in the Evelyn photoelectric colorimeter. The combination of light filters, Rubicon No. 4785 and Wratten No. 75, cemented in "C" glass, permits the maximum transmission of light in the 490 to 500 mμ range. This is the most selective range for the Jaffe picrate reaction.

It was found necessary to use the bright light of the instrument. As a result a slightly different manipulation was adopted than when the dim lamp is used. A blank solution containing 1.0 cc. of 2 N HCl, 1.0 cc. of 2 N NaOH, 8.0 cc. of water, and 5.0 cc. of alkaline picrate was allowed to stand for 10 minutes after the addition of the alkaline picrate. Meanwhile the proper light filters had been placed in the colorimeter and the dim lamp turned on. The rheostats were adjusted until the galvanometer read about 55. At the end of the 10 minute period, the colorimeter tube with its contents was placed in the instrument, the bright light turned on, and the rheostats adjusted until the galvanometer read 100. The bright lamp was then turned off, the tube removed, and the new galvanometer reading with the dim lamp observed. This new reading is called the "center reading." This manipulation was repeated for 1.5 minutes in order to secure a constant center reading with the particular instrument used. This probably is not due to any color change in the solution, but to varying the light intensity and the heat effects arising therefrom. The same procedure of removing the tube to check the center reading was used for all creatinine measurements, and it gave satisfactory and reproducible results over extended periods of time. The advantage of the center reading is that it serves as a reference point of light intensity, and eliminates numerous blanks in a particular set of creatinine determinations. It should be determined with each set of creatinine values.

The reagent picric acid was prepared from Eastman's No. 210 by purifying twice by Benedict's method (16). The 1.2 per cent solution was made without the application of heat by trituration with water at room temperature. This solution was freshly prepared 24 hours before each run.

The alkaline picrate solution was made by mixing 5 volumes of the 1.2 per cent picric acid and 1 volume of exactly 2.5 N NaOH and allowing to stand for 15 minutes before use. It was found safe for use up to 6 hours after preparation.

The creatine was converted into creatinine in special conversion tubes. The tubes of 25 cc. capacity were made from No. 24/40 interchangeable ground glass connections and provided with stoppers, No. 24/25.[1] The stoppers and tubes were fitted with glass hooks for the application of springs or wires. For the conversion of creatine to creatinine the standard solution or unknown was measured into the dry tube, water added to dilute to 5 cc., and after the addition of 1 cc. of 2 N HCl the tubes were stoppered, securely wired, and autoclaved at 20 pounds pressure for 45 minutes. The autoclave was allowed to cool until the pressure returned to the atmospheric before it was opened. After the tubes had cooled to room temperature, 1 cc. of 2 N NaOH and 3 cc. of water were added and the mixture stirred with a fine stirring rod. For the color development, 5 cc. of the alkaline picrate solution were added and 10 minutes after thorough stirring with a glass rod the mixture was transferred to the colorimeter tube and read in the colorimeter.

The standard creatinine curve was prepared by measuring the standard creatinine solution directly into the colorimeter tube, adding 1 cc. of 2 N HCl, 1 cc. of 2 N NaOH, and diluting to 10 cc. with water. The acid and base were added in order to have the same salt concentration as in the creatine estimation. The 5 cc. of alkaline picrate were added and 10 minutes after thorough mixing the tubes were read in the colorimeter. Ranges of 0 to 0.05 mg. of creatinine and the equivalent of creatine were run. The standard curve is given in Fig. 1. Obviously Beer's law holds and the creatine is quantitatively converted into creatinine. The accuracy of the curve is ±2.2 per cent as a maximum deviation from the mean in the range given.

Animal Experiments—Sixteen male rats of the same age from our inbred colony were used. Eight were castrated at 3 months of age. All were placed in metabolism cages maintained at 22°. Two rats were kept in each of eight cages on a constant diet for 2 months prior to the urine analysis. The diet consisted of commercial casein 18 per cent, corn-starch 53, inorganic salt mixture (17) 4, butter fat 8, cod liver oil 2, dried brewers' yeast 15. Each day the cages, funnels, and volumetric flasks for urine collection

[1] Scientific Glass Apparatus Company.

were thoroughly cleaned at the same hour in an attempt to keep all conditions constant. On urine collection days, the cages, false bottoms, and funnels were washed thoroughly with distilled water. The feces-free washings were collected in a 500 cc. volumetric flask, made up to volume with water, and filtered. Aliquots of the filtrate were taken for creatinine and total creatinine determinations.

After a control period on· creatine and creatinine excretion, creatine amounting to 40 mg. per kilo of body weight was administered orally to each rat in two divided doses each day. The
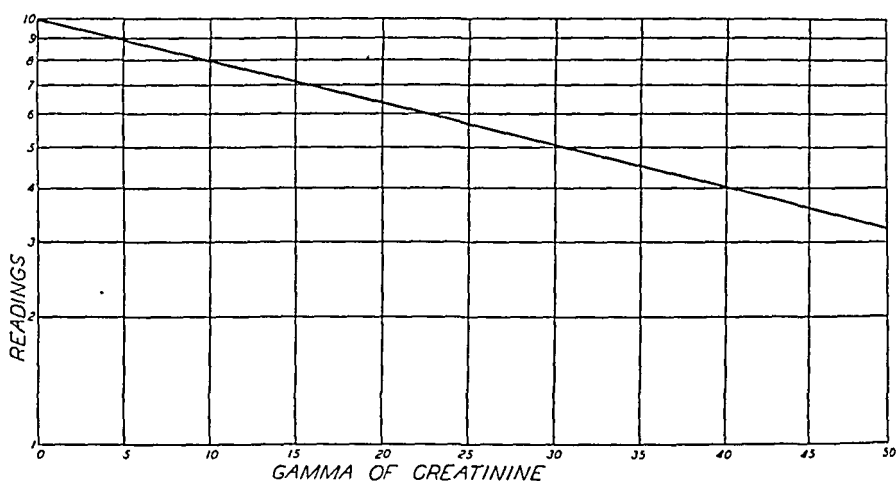
FIG. 1. The standard creatine-creatinine curve for the Evelyn photoelectric colorimeter. $C = (2 - \log G)/K_1$ where G = colorimeter reading and $K_1 = 9.70$.

creatine solution was administered by a stomach tube, which consisted of a No. 14 1½ inch blunt metal tube attached to a hypodermic syringe. The tube was placed down the rat's esophagus and the water solution of creatine forced into the stomach. This allows the creatine to be applied in a manner which simulates that of normal feeding and causes it to follow the normal path of absorption from the intestine. Thus an intense creatinuria was produced. After 5 days of creatine administration the rats approximated a constant body weight. Thereafter, throughout the entire experiment the corresponding amount of creatine was given. Thus, as the rats gained weight the creatine administered fell somewhat below the value of 40 mg. per kilo of body weight.

Creatine alone was given for 15 days until an intense creatinuria was produced. On the 16th day, in addition to the continued ingestion of creatine, each rat was injected daily with 900 γ of testosterone propionate in sesame oil for 14 days. During the androgen injections the urine was pooled and extracted for androgenic assay. This and a similar previous experiment resulted in no androgenic activity being excreted by the normal and castrated rats, although a total of 13,440 I.U. of testosterone propionate had been administered.

After the 14 day period of testosterone propionate and simultaneous creatine administration, both treatments were discontinued and the creatine and creatinine excretion observed for 11 more days.

During the entire experiment the body weights were recorded for correlation studies.

The creatinine and total creatinine determinations were made by diluting the 24 hour specimen of urine to the equivalent of 1 liter, and using 1 cc. aliquots for duplicate analyses. The amount of non-creatinine chromogenic material in the urine was determined before and after autoclaving by the use of the Dubos-Miller (14) specific creatine- and creatinine-destroying cultures. It amounted to approximately 1 mg. of creatinine equivalent per day and was constant before and after autoclaving, thus eliminating the necessity for any corrections to be applied to the total creatinine determination.

Figs. 2 and 3 show the values obtained for the body weights, and creatine and creatinine excretion in normal and castrated rats respectively. In the normal rats the creatinuria during the control period is of a very low order. Creatine administered orally causes intense creatinuria. Testosterone propionate injection during creatine ingestion lowers the creatinuria. Simultaneously with the decrease in creatine excretion there is an increase in body weight which approximates a new high level. After this level is reached, the creatinuria again increases. In the fourth period, when the androgen and creatine administrations were discontinued, there was a slight decrease in body weight and a return of the creatine excretion to the pretreatment level. The values for the castrated rats give a similar set of curves, but the changes in creatinuria and body weight are much greater than in the normal

rats, and the attainment of the new high level in body weight requires about 3 days longer than for the normal animals.

To show more clearly that the castrated rats do excrete less of the ingested creatine than the normals during androgen administration, the average values of creatine excreted are expressed as per cent of creatine fed, and plotted as maximum and minimum
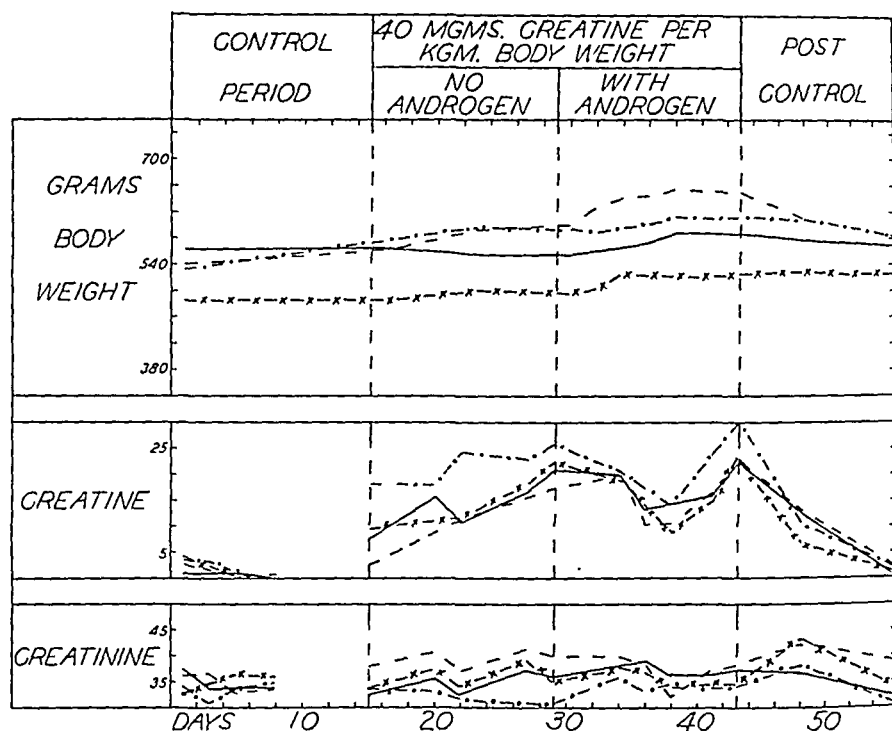
FIG. 2. The urinary creatine and creatinine from adult male rats. The solid line represents the data for Pair 3; the dash line, Pair 4; the dot and dash line, Pair 7; the cross and dash line, Pair 8. The creatine and creatinine values are expressed as mg. per kilo of body weight.

deviation from the mean excretion values. Fig. 4 shows these relationships. Although there is a greater retention of ingested creatine by the castrated rats, both types of animals react in a similar fashion to the creatine and androgen treatments. The retention differs quantitatively but not qualitatively. Thus, there is a striking parallelism in the creatine excretion curves. Also the ratio of body weight gains of castrates and normals and the ratio

of the creatine retained are 1.7 and 1.45 respectively. That is, increased body weight paralleled the increased creatine retention.

Subsequent to the urinary creatine investigations, an estimation of muscle creatine was undertaken on the same rats. They were kept on the stock diet for 36 days after the previous urine experiments. At the end of that time the animals were divided into four groups. One set each of normals and castrates received
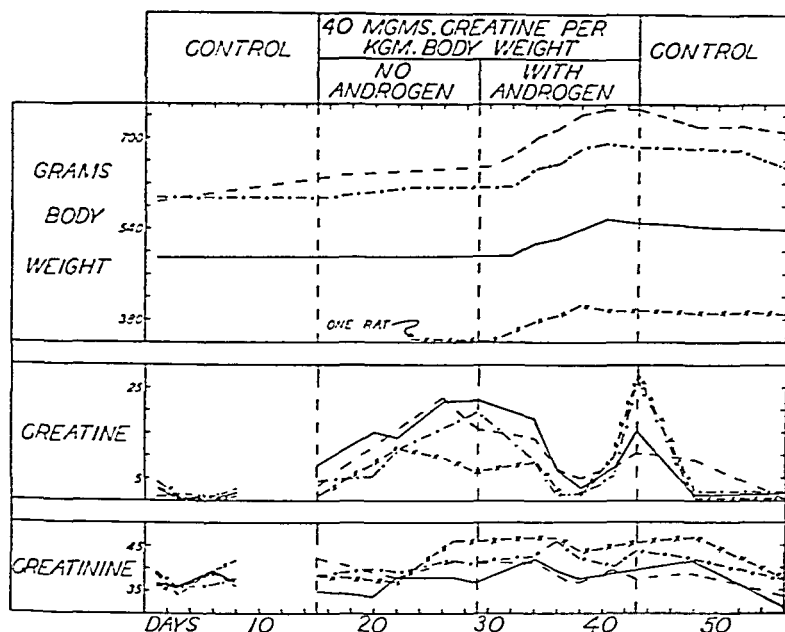
FIG. 3. The urinary creatine and creatinine from castrated adult male rats. The solid line represents the data for Pair 1; the dash line, Pair 2; the dot and dash line, Pair 5; the cross and dash line, Pair 6. The creatine and creatinine values are expressed as mg. per kilo of body weight.

40 mg. per kilo of body weight of creatine orally each day, while the other sets of normals and castrates received 900 γ of testosterone propionate in sesame oil daily in addition to the ingested creatine. The creatine and androgen were administered for 19 days after which time a constant elevated body weight was reached in the androgen-injected animals. The rats were then sacrificed and the gastrocnemius muscle removed from both hind
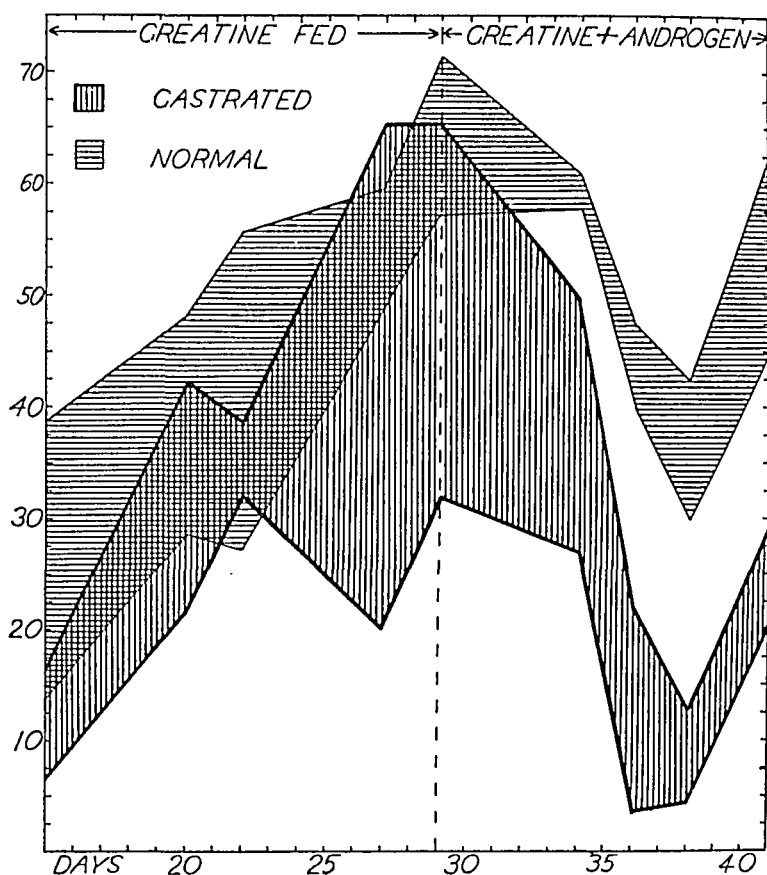
FIG. 4. The urinary creatine as per cent of creatine fed. The high and low values of the mean deviation from the average are plotted.

TABLE I

Per Cent of True Total Creatinine in Dry and Fat-Free Muscle Tissue of Albino Male Rats Receiving 40 Mg. per Kilo of Body Weight of Creatine Orally per Day

	Control		900 γ testosterone propionate daily for 19 days	
	Castrated	Normal	Castrated	Normal
Maximum...................	2.00	1.99	2.36	2.18
Minimum....................	1.90	1.90	2.07	1.92.
Average....................	1.95	1.95	2.18	2.02

legs for creatine determinations. In order to obtain a representative sample of tissue, the muscles were immediately frozen on

solid carbon dioxide and pulverized in a previously chilled iron mortar. The powdered tissue was stored at $-5°$ for a short time until aliquots were taken for total creatinine, moisture, and fat determinations. The total creatinine was determined by the method of Miller, Allinson, and Baker (18) by the use of the Dubos-Miller specific creatine- and creatinine-destroying cultures. A standard curve for creatinine concentration *versus* per cent of light transmission was produced under conditions identical with those used for the tissues. Table I shows the total creatinine values in the gastrocnemius tissue. These figures indicate that there is no significant difference in the muscle creatine between normal and castrated rats with and without androgen administration, but with a liberal supply of exogenous creatine.

DISCUSSION

The gain in body weight as a result of androgen therapy is in accordance with the observations of Korenchevsky, Dennison, and Brovain (19) on castrated rats, and Kenyon, Sandiford, Bryan, Knowlton, and Koch (13) in eunuchoids. The latter group believe that from one-seventh to one-half of this gain probably is due to protein being laid down as indicated by the nitrogen retention studies, but that a considerable amount of the increase is due to water and sodium retention. Similarly, Thorn and Harrop (20) have found that sodium and its associated water are retained in the normal dog during the administration of estrone, estradiol, progesterone, pregnanediol, and testosterone.

Gaebler and Bartlett (21) have produced decreased exogenous creatinuria in adult female dogs by the administration of antuitrin growth preparation. Simultaneously, there resulted an increase in nitrogen retention and body weight. Here the decrease in exogenous creatinuria also parallels body weight gain. This may be an instance of a substance other than a sex hormone which has a similar effect on body weight and creatinuria. However, it is possible that the growth hormone preparation which these workers used was not free from gonadotropic factors and hence may have produced the changes through the gonads. The fact that pre-pubertal growth is associated with a constant creatinuria in both sexes, and that postpubertal growth results in a diminished creatinuria, may be of significance in the interpretation of these results.

The belief held by some investigators, that creatine excretion in men is an indication of a hypogonad function, is not confirmed by the studies in rats. Prior to feeding of creatine no significant difference in creatinuria existed between the normal and castrated rats. During creatine feeding the creatinuria of the castrated rats varied from 12 to 22 mg. per kilo of body weight, whereas the normal rats excreted 20 to 26 mg. per kilo of body weight just prior to androgen injection. Fig. 4 shows that throughout the creatine feeding period the castrated rats tended to excrete less of the ingested creatine than the normal animals.

Another question which requires more investigation is the creatinuria of normal men. It has been generally accepted that normal men do not have a creatinuria, but evidence is accumulating which indicates that this belief is not well founded. Taylor and Chew (22) found from 0 to 196 mg. of creatine nitrogen in the urine of fifteen adult males. Other unpublished observations also indicate that normal men may excrete creatine in the urine in varying amounts.

Since it is generally believed that creatine when ingested is in part stored by the muscles, and about 98 per cent of the body's creatine resides in the musculature, it seems possible that the increased exogenous creatine retention observed in these experiments, paralleling body weight gain, represents an increased muscle tissue production under the influence of testosterone propionate. This view is supported by the observations of Papanicolaou and Falk (23) who showed that the temporal muscles of male guinea pigs are larger than those of females. They also observed that in male and female castrates a muscular hypertrophy was produced by the administration of testosterone.

The observation that the castrated rats had a body weight gain of 1.7 times that of the normals is in agreement with Kochakian and Murlin's (8) reports on the effects of androstenedione upon nitrogen retention in castrated and normal dogs. Their experiments showed very little if any nitrogen retention on the part of the normal as compared with the castrated dog. As a result of this, they felt that an animal with normally functioning gonads is being supplied with sufficient hormone to maintain at least its accessory sex organs in a normal physiological condition, and that further androgen is either not utilized or is met with a compensating set of factors. The doses used in their dogs were low as com-

pared with levels used in the rats.   Therefore, in the rats it appears that in the normal animal, although it does gain weight, there is some compensating factor at work which decreases the effect of excess androgen before a similar condition is brought about in the castrated animal.   This is indicated by the lower gain in weight which reaches a maximum about 3 days earlier than in the castrates.

SUMMARY

The quantitative estimation of creatine and creatinine has been studied by the use of the Jaffe reaction, Evelyn photoelectric colorimeter, and the Dubos-Miller specific creatine- and creatinine-destroying cultures.   Certain modifications of the determination have been made and applied to biological materials.

Adult male rats which have been in a castrated condition for 2 months do not develop a distinct creatinuria other than the insignificant and apparent creatinuria existing prior to operation.

Daily injection of 900 γ of testosterone propionate caused an increase in body weight and a decrease in excretion of exogenous creatine.   The body weight gain and the decrease in creatinuria were greater in the castrated than in the normal animal.

The castrated and normal animals react in a similar fashion to exogenous creatine and testosterone propionate.   During testosterone propionate and creatine administration the changes in creatinuria parallel body weight change until the body weight reaches a high level.   At this level an intense creatinuria reappears even though androgen administration is continued together with the ingestion of creatine.   When the administration of androgen and creatine is discontinued, the creatinuria falls to the insignificant values of the pretreatment level.

The muscle creatine content of normal and castrated rats with and without androgen treatment, but with a liberal supply of exogenous creatine, shows no significant difference.   It would appear that the muscle tissue of the castrated rat is normal with respect to creatine content under the conditions of the experiment.

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THE ASSIMILATION OF AMMONIA NITROGEN BY THE TOBACCO PLANT: A PRELIMINARY STUDY WITH ISOTOPIC NITROGEN

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When ammonium chloride that contains an excess of nitrogen of atomic weight 15 is administered to plants grown in culture solution, it is possible, by means of isotope analysis of suitable fractions of the tissues, to detect certain details of the course of assimilation of this nitrogen. Reactions that are roughly divisible into two categories are to be anticipated; first, those that represent normal increase of tissue components associated with growth and, second, those that represent interactions between substances already present and substances that contain the newly acquired nitrogen whereby the isotope is introduced into the former.

Reactions of this second type, which result in a shift of nitrogen from one compound to another, have been studied in mature animals in nitrogen equilibrium. The prompt introduction of isotopic nitrogen into many of the amino acids of the body proteins has been observed when isotopic ammonium salts or amino acids are administered in the food of such animals (1, 2). The replacement of the protein nitrogen by the dietary nitrogen is clearly a result of continuous chemical reactions in which the body proteins are normally concerned. The demonstration of this type of chemical change is of especial importance, since it represents a form of metabolic activity the existence of which has only recently become appreciated in animals and of which there are few if any records in plants.

Plants are less favorable objects for this demonstration than animals. At maturity, when the reproductive cycle is completed, senescence rapidly follows; with herbs, death of the entire plant may occur or, in other species, there may be a withdrawal of metabolic products into the root system for storage against the growth of the following season. A protracted period during which the situation is analogous to that of a mature animal in nitrogen equilibrium is not usually attained, since plants do not excrete nitrogen but respond to its administration by growth or by the development of the reproductive organs.

The introduction of nutriment into the plant system also presents problems somewhat different from those experienced with the animal. The greater part of the normal nutriment absorbed by the roots consists of inorganic ions. In the absence of suitable salts, growth diminishes sharply[1] or ceases, and a condition is rapidly set up in which the plant utilizes its stores of inorganic material, nitrogen compounds, organic acids, and carbohydrates in ways different from the normal.

Nevertheless it has seemed desirable to test the behavior of plants to which isotopic ammonium ions were administered through the roots in order to provide an indication of the types of reaction that occur. It was found that this nitrogen is rapidly absorbed and transported to all parts of the plant. Within a short time, the isotope could be detected in the amide groups of asparagine and glutamine and also in the water-soluble tissue components that are precipitated by mercuric acetate and sodium carbonate (Neuberg reagent); that is, in the fraction that includes free amino acids and certain basic substances. In addition, considerable amounts of isotopic nitrogen were found in the proteins of the leaves, stalk, and root. The picture presented is one of great chemical reactivity, the amides and amino acids presumably behaving as intermediates in the synthesis of proteins from the newly acquired ammonia.

EXPERIMENTAL

Owing to the small supply of isotopic nitrogen available, experimentation was necessarily severely limited in scope. The

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[1] Plants are especially sensitive in their response to the administration of nitrogen; when any deficiency in the supply of nitrogen is made good, renewed growth is to be anticipated at almost any stage in the life history.

culture solution employed had the composition KH_2PO_4 0.00325 M, $Ca(NO_3)_2$ 0.00215 M, $MgSO_4$ 0.00105 M, NH_4Cl 0.0043 M, $CaCl_2$ 0.00215 M. To this were added traces of boron and of heavy metals and 0.5 part per million of iron. The plants were grown according to the Shive technique, the solution being continuously renewed at such a rate that the effluent was maintained approximately at pH 5.0. For the experiment with isotopic nitrogen, a single tobacco plant 41 days after transplantation of the seedling was

TABLE I

Effect of Administration of Ammonium Chloride with 1.21 Atom Per Cent N^{15} Excess on Certain Nitrogenous Components of Tobacco Plant Tissues

A single plant 41 days from the seedling stage was treated for 72 hours. The data are expressed as atom per cent N^{15} excess.

	Leaf		Stalk		Root	
	Found	Calculated for 100 per cent N^{15} in NH_4Cl administered	Found	Calculated for 100 per cent N^{15} in NH_4Cl administered	Found	Calculated for 100 per cent N^{15} in NH_4Cl administered
Protein N..............	0.099	8.2	0.184	15.2	0.222	18.4
Ammonia N.............	0.260	21.5	0.275	22.7	0.779	64.3
Amide N...............	0.217	17.9	0.286	23.6		
Water-soluble N pptd. by Neuberg reagent........	0.184	15.2	0.225	18.6		
Water-soluble N not pptd. by Neuberg reagent......	0.123	10.2	0.108	8.9		
Volatile base N...........	0.030	2.5				
Alcohol-soluble N not soluble in water..:........	0.078	6.4	0.104	8.6	0.200	16.5
Water-soluble N exclusive of ammonia N..........					0.414	34.2

transferred to an otherwise similar culture solution in which ammonium chloride with 1.21 atom per cent N^{15} excess was substituted for the ordinary ammonium chloride; 3 days later the plant was dissected into leaves, stalk, and roots and the tissues were weighed and dried at 80° for analysis. The analytical methods employed have been described in previous papers (3, 4) and the fractions prepared from each tissue are shown in Table I.

Table I gives the results of the isotope analyses expressed in atom per cent N^{15} excess in each fraction and also the values cal-

culated from these results that would have been obtained if 100 per cent isotopic nitrogen had been used instead of 1.21 atom per cent excess material. This calculation is in accordance with the method of presentation in other recent papers in which N^{15} has been used as a biological tracer element. Each figure gives directly the percentage of the nitrogen that had been derived from the ammonia of the culture solution during the experimental period.

The provision of control plants and of a sufficient number of experimental plants so that an accurate estimate could be made of the rate of growth of this plant under the experimental conditions was not possible. However, earlier studies of the rate of growth of tobacco plants in the field (3) have shown that individuals of the size and age of the present plant might be expected to have

TABLE II

Protein Nitrogen in Tobacco Plant Treated with Isotopic Ammonia for 72 Hours

	Total N	Amount of N^{15}
	m.eq.	*microeq.*
Leaf protein N.......................	29.55	29.2
Stalk " "	3.77	6.9
Root " "	5.32	11.8
Total................................	38.64	47.9
Administered NH_3-N...................	10.7	129

acquired approximately 25 per cent of their dry weight and nitrogen in the preceding 3 days. During the 72 hour experimental period, the plant absorbed 0.1498 gm. of ammonia nitrogen and 0.0737 gm. of nitrate nitrogen as determined from analysis of the culture solution. At the end, it contained 1.069 gm. of nitrogen of which 0.725 gm. was found in the leaves, 0.197 gm. in the stalk, and 0.147 gm. in the roots. Evidently, therefore, 20.9 per cent of the nitrogen had been absorbed during the previous 72 hours; of this the ammonia of the culture solution contributed two-thirds. A rough estimate of the rate of growth in the experimental period is thus provided.

Table II shows the quantities of protein nitrogen, together with the amounts of isotopic nitrogen each tissue protein contained,

calculated from the ratios in Table I. Since the ammonia nitrogen administered contained 1.21 atom per cent N^{15} excess, the whole plant had received 129 microequivalents of N^{15}. The proteins had then acquired 47.9 microequivalents and, accordingly, 37 per cent of the absorbed isotopic nitrogen had found its way into the proteins of the tissues. This proportion is almost certainly significantly greater than could have been expected from the growth of new tissue and the synthesis of new protein during the experimental period, and strongly suggests the occurrence of chemical reactions by means of which proteins present from the start of the experimental period obtained isotopic nitrogen from other components of the cells.

DISCUSSION

Consideration of the data in Table I suggests that chemical reactions in which ammonia was concerned were especially marked in the roots. About 34 per cent of the water-soluble nitrogen, exclusive of ammonia, was derived from the ammonia of the nutrient solution during the experimental period. If this were due to the increase in number of organic molecules alone, it would be necessary to assume that one-third of this material had been developed within 72 hours. This is hardly likely and, as an alternative explanation, it may be suggested that reactions took place whereby newly acquired nitrogen was introduced into the water-soluble nitrogenous components of the cells. The high concentration of isotopic ammonia present in the roots at the end of the experimental period may also be pointed out in support of this view.

The concentrations of isotopic nitrogen in the ammonia, the amide nitrogen, and the nitrogen of the Neuberg reagent precipitate (5) derived respectively from the stalk and leaves were substantially the same and of a magnitude not greatly different from that to be anticipated from the increase due to the growth of the plant. There is no clear evidence from these quantities alone that other reactions occurred by which nitrogen from the nutrient was introduced into compounds already present.

The concentration of N^{15} in the nitrogen of the proteins of the stalk and leaves, particularly in the latter, was definitely lower than that observed in the ammonia and amide nitrogen of these

respective tissues. It might be assumed from this that no re-
actions occurred in which the proteins shared, save the synthesis
of protein due to growth. On the other hand, as is shown in
Table II, when the actual quantities of protein are taken into con-
sideration, appreciably more isotopic nitrogen was found in the
proteins than can be reasonably accounted for in terms of growth;
reactions that involve chemical interaction of nitrogen as between
protein and soluble cell components apparently did occur.

In order to provide clearer evidence of this, a sample of the
protein of the leaf tissue was hydrolyzed and several amino acids
were isolated. The methods employed were substantially those
described by Schoenheimer, Ratner, and Rittenberg (2). Purified
specimens of arginine, histidine, glutamic acid, and aspartic acid

TABLE III

N^{15} Concentration (Atom Per Cent Excess) in Amino Acids and Amino Acid
Fractions from Crude Protein of Leaves of Tobacco Plant to Which
Isotopic Nitrogen Had Been Administered

Total protein..	0.099
Arginine..	0.086
Histidine...	0.090
Glutamic acid..	0.128
Aspartic "	0.113
Copper salts insoluble in water and in methanol.........	0.104
" " " " " but soluble in methanol..	0.100
Remaining amino acids.....................................	0.096

were secured for isotope analysis and, in addition, fractions of
mixed amino acids obtained by the use of copper salts were ex-
amined. The data are given in Table III. The bases and the
mixed amino acids in the copper salt fractions all had substantially
the same proportion of isotope and this was not greatly different
from that of the whole protein. This evidence alone would merely
indicate the synthesis of new protein corresponding to the growth
of the tissue. The glutamic and aspartic acids, however, con-
tained a significantly larger proportion of isotopic nitrogen. To
account for this in terms of growth alone would involve the un-
likely assumption that the protein synthesized during the experi-
mental period differed in amino acid composition from that present
at the start in that it contained a much higher proportion of glu-

tamic and aspartic acids. If the acquisition of isotopic nitrogen by the protein were solely due to growth, all the amino acids should have substantially the same isotope ratio. The augmented ratios observed for the glutamic and aspartic acids clearly imply that these amino acids interacted with other nitrogen or at different rates than the other amino acids and provide an example from plant tissues that is closely analogous to previous observations on the metabolism of proteins in animals (2). Some process *in addition* to mere growth of the plant with its attendant synthesis of protein must have occurred in order to account for the augmented replacement of the nitrogen of the glutamic and aspartic acids. This process must have been followed by the synthesis of these acids into the protein molecule and must have been preceded by the release of glutamic and aspartic acids or of their potential equivalents from protein already formed.

The relatively low concentration of the isotope in the nitrogen of the substances that were not precipitated by the Neuberg reagent suggests that these components play a less active rôle in the nitrogen metabolism. The quantity of nitrogen was by no means inconsiderable, the order of magnitude being similar in each case to that of the nitrogen precipitated by the Neuberg reagent. Much of it, however, consisted of nitrate nitrogen which does not undergo spontaneous exchange with ammonia nitrogen (6).

The nitrogen of the volatile base (exclusive of ammonia) in the leaf tissue, a part of which was identified as nicotine, contained very little isotope. A low order of reactivity of the nitrogen of this compound is suggested. That part of the nitrogen of the leaf and stalk insoluble in hot water but extracted by hot alcohol, a fraction which contained the chlorophyll, also showed only minor evidences of replacement.

No attempt to account for the detailed mechanism whereby so large an amount of isotopic nitrogen was assimilated into the proteins of this plant can be advanced at the present time. However, it may be worth while to point out that, if the proteins of plant cells undergo continuous decomposition and resynthesis at a high rate, as is envisaged by the views of Gregory and Sen (7) on the mechanisms involved in plant tissue respiration, the rapid introduction of isotopic nitrogen into the proteins follows as a logical consequence.

Buckwheat Experiment

A similar experiment conducted with twenty-two buckwheat plants transferred for 47 hours, at the time of initial blossoming, to a culture solution identical with that employed for the tobacco plant experiment gave closely similar results. Some of the data have already been submitted in a preliminary communication (8). Owing to the small quantities of tissue available, the full data were less satisfactory than those from the tobacco plant and are therefore not given in detail. Isotopic nitrogen was found, however, in all fractions, being present in high concentration in the proteins and in the amide nitrogen and water-soluble substances precipitated by the Neuberg reagent from extracts of the leaves and stalks. As in the tobacco plant experiment, it was present in only low concentration in the filtrates from the Neuberg reagent precipitates. The effects on the stalk constituents were strikingly more intense than were those on the leaf constituents, though the actual quantities of the several nitrogenous substances in the stalks were much smaller than in the leaves.

SUMMARY

When ammonium chloride that contains nitrogen of atomic weight 15 is administered for a short period to rapidly growing plants, the isotope can be promptly detected in all parts of the tissues. The ammonia absorbed is rapidly assimilated into the nitrogen of amides and amino acids and into the proteins. The concentration of isotope in the nitrogen suggests greatest intensity of assimilation in the roots and least in the leaves; on the other hand the greatest quantity of isotopic nitrogen was found in the leaves. Much of the chemical change can be accounted for as a result of growth during the experimental period, but the *quantity* of isotope found in the proteins of the tissues was appreciably in excess of that to be expected from growth alone. This excess is apparently the result of continuous chemical interaction between the nitrogen of the tissue constituents and that of the nutrient. In the course of these reactions isotopic nitrogen is introduced into the proteins. This is held to furnish an example of the *normal* process of nitrogen assimilation. There is a close and striking analogy to the type of continuous chemical reaction in which the

tissue proteins of mature animals in nitrogen equilibrium have already been observed to be concerned.

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A SIMPLE ULTRAFILTRATION APPARATUS

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The ultrafiltration tube shown in Fig. 1 has proved convenient to several workers in this laboratory in showing that bilirubin is and that ascorbic acid is not bound to the plasma proteins and in other experiments.

The membrane used is Visking sausage casing size 30/32, sold in 500 foot rolls. This is soaked 20 minutes, knotted, invaginated, and filled as shown (the knot may be omitted). It is then wrapped with a single layer of the commonest variety of hard tissue paper, fitted snugly into the tube, and fastened with a rubber band. It is centrifuged at a speed gradually increasing to 1500 R.P.M. for 5 minutes, and then at 3500 R.P.M. for as long as desired. 10 cc. of plasma should yield about 3 cc. of ultrafiltrate in an hour. Occasionally a small amount of protein passes through. This can be removed by repeating the filtration.

The Pyrex tube has a porous Pyrex shelf of fine porosity for strength. The lower end of the upper half of the tube is made hemispherical internally to fit the casing closely. The dimensions given are those of a tube which fits the longer brass cup of the commonest large centrifuge. The hole in the lower part of the tube allows withdrawal of the filtrate with a pipette.

6"

POROUS
GLASS
SHELF

RUBBER
BAND

3"

TISSUE
PAPER

⊢ 1 5/16" ⊣
EXTERNAL
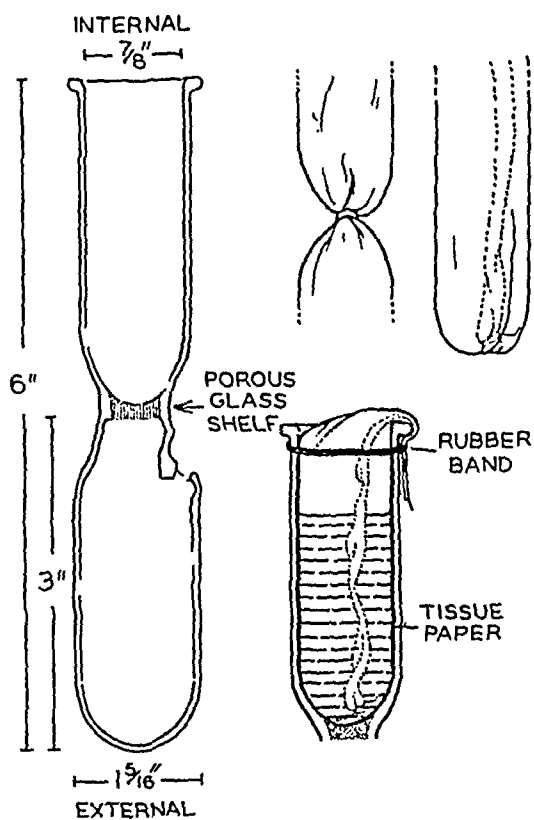
FIG. 1

METALLOPORPHYRINS

I. COORDINATION WITH NITROGENOUS BASES. THEORETICAL RELATIONS*

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In this series of studies we shall contribute to the systematic description of the behavior of metalloporphyrins in their coordination with nitrogenous bases.

The first method of attack will be the potentiometric. The results will be supplemented by spectrophotometric measurements.

Conant and his students (1, 2) initiated the potentiometric exploration of systems containing heme and a base that coordinates with heme in both the oxidized and reduced states. Such studies have been extended by Barron and Hastings (3) and particularly by Barron (4). Also important contributions to our knowledge of these complex systems have come by way of spectrophotometry, notably at the hands of Hogness, Zscheile, Sidwell, and Barron (5). The assembly of facts thus made available clarifies relations discussed in the earlier, enlightening papers of Anson and Mirsky (6) and Mirsky and Anson (7).

We shall deal with several systems containing not only different bases but metalloporphyrins composed of different metals and different porphyrins; therefore we require an adaptable nomenclature. We shall report the evidence of considerable complexity; therefore we think it wise to outline with care both the presumptions and the restrictions of a theoretical approach. We shall

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* A condensation of the theoretical parts of dissertations the experimental data of which are reported in the following papers by John Fuller Taylor, T. Harrison Davies, and Carl S. Vestling.

have to submit our data to various sorts of analysis.   Since that of any one sort will be repeated, we shall conserve space by devoting the greater part of this paper to the development of propositions that can be submitted to experimental tests and that may be referred to later by numbers.   Exploratory measurements, detailed in the dissertations of Taylor (8), Davies (9), and Vestling (10) are summarized in the following papers by these authors.

Nomenclature

Several of the terms commonly employed in this field stem from those which arose during the early study of the prosthetic group of mammalian blood pigments and are not etymologically good when applied to substances containing metals other than iron and porphyrins other than protoporphyrin IX.   Some attempts at reform have left the nomenclature in more or less confusion.   We had adopted a simple nomenclature when Drabkin (11) proposed essentially the same system.

A compound formed from a porphyrin and a metal ion is called a metalloporphyrin.   The metal and its state may be designated by expressions such as *ferro, cobalti*, etc., as in *manganomesoporphyrin IX*.   A compound formed from a metalloporphyrin and another substance is designated by prefixing the name of the coordinating substance to that of the metalloporphyrin, as in *nicotine ferriprotoporphyrin*.   In running commentaries, where the specific substance is clearly understood, a compound such as cyanide ferrohematoporphyrin may be spoken of as a base metalloporphyrin, *base* here being used in a wide sense.

Our symbols will refer to nitrogenous bases as coordinating substances but we do not wish to imply that only nitrogenous bases are able to coordinate with metalloporphyrins.

It will be assumed that the preparative procedures and certain identifying tests, which confirm what others have described, suffice to identify our porphyrins.   If there be occasion to define a porphyrin quite specifically, both the name and the number given in Fischer's (12) system will be used, as in the name *mesoporphyrin IX*.

Scope and General Definition of Problems

Experience in this laboratory has confirmed our guess that some of the general features of these systems will be revealed by

modifying properties pertaining to one or another class of the components. Accordingly, porphyrin, metal, and coordinating base have been changed independently.

What was not foreseen is that when components are modified each of the systems so far studied has some property that restricts severely the range of experimental study. Accordingly the data require some piecing together if one is to obtain a general formulation of the equilibria and a tentative picture of the coordination compounds that are involved. In attempting this we find it essential to have in view the types of reaction that are to be met and equations formulating those under special study. Indeed, both the older data and much of that gathered in our first experiments are inadequate, because there were lacking those formulations that could have indicated critical relations. Also, such criticism of our final conclusions as may be made should involve a scrutiny of the basic assumptions.

The phenomena observed to date show that the following types of reaction occur in systems containing a metalloporphyrin and a coordinating base. The list is restricted to reactions that are pertinent to the immediate subject. As occasion demands, a more detailed formulation will be constructed with the accumulating evidence and a few postulates, and this will serve to account for the ionic charges not mentioned in the following primitive formulations.

1. Reversible association between porphyrin and reduced metal ion.

Example—Ferrous ion + protoporphyrin $\rightleftharpoons$ ferroprotoporphyrin

It will be recognized that this type of reaction, studied by Fischer, Treibs, and Zeile (13), is involved in the preparation of porphyrins from metalloporphyrins and of metalloporphyrins from porphyrins and metal ions. Except under the special conditions which Vestling (10) describes in his study of preparative methods, it will be assumed that the equilibrium state of this reaction is far "to the right." This assumption seems to be generally accepted for the alkaline water, and water-alcohol solutions such as were used in our studies and accordingly we have not examined it with the care that is desired ultimately.

That metal ions combine with porphyrins containing no acidic group other than $>$NH is the most convincing of the evidences

that the predominating species of the metalloporphyrins having carboxyl groups are not normal salts of the carboxylic acids. Other evidence has confirmed the belief that they are coordination compounds involving the pyrryl nitrogens. This will be so far the tacit assumption that our formulations will provide for only one sort of metalloporphyrin, which we have already called a "coordination compound."

2. Reversible oxidation and reduction of the metalloporphyrin, if the metal component be of appropriate type.

Example—Cobaltimesoporphyrin + electron $\rightleftharpoons$ cobaltomesoporphyrin

It seems unnecessary to review the evidence that there is a preferential response to oxidizing and reducing agents, and that when these agents are suitably apportioned the first response is determined by the state of the metal component and that groups characteristic of the porphyrin alone are but slightly affected, if at all. Since each of the metals that we have used is such that 1 equivalent is concerned in the reduction of the quantity of metalloporphyrin containing a gm. atom of metal, formulations will be simplified by retaining this specification.

3. Reversible dissociation of hydrions (or hydroxyl ions).

Since porphyrin, metalloporphyrin, coordinating base, and base metalloporphyrin may have distinctive, or, in some cases, common groups with different acidic dissociation constants, there are numerous theoretical possibilities for the integration of the free energies of acid ionization and of hydrion dilution with the free energy of the ideal oxidation-reduction process. Actually, of course, all may be integrated in what may be called a continuum (Clark (14)). We may simplify matters greatly by anticipating the evidence that under the limited conditions of the measurements the detected, dissociable hydrions (or hydroxyl ions) pertain to the coordination center rather than to groups characteristic of the porphyrin alone or of the coordinating base alone. Ionizations pertaining to the latter two sorts of groups have to be considered under other circumstances. For the most part the range of pH used in the experimental studies was such that the carboxyl groups of the porphyrin can be *assumed* to be ionized.

4. Reversible association between oxidized metalloporphyrin and a nitrogenous base (including cyanide ion) and between reduced metalloporphyrin and the same base.

Examples—

Manganomesoporphyrin + r pyridine ⇌ pyridine$_r$ manganomesoporphyrin

Manganimesoporphyrin + q pyridine ⇌ pyridine$_q$ manganimesoporphyrin

It is the difference between the dissociation constants of the oxidized and reduced base metalloporphyrins that makes possible the potentiometric evaluation of these constants. This is the unique feature of the following papers the mathematical introduction of which is given in this paper.

5. Formation of polymers of metalloporphyrin or of a base metalloporphyrin.

On the one hand there will be given evidence of the existence of dimers in certain instances. On the other hand this calls for equations that are very complex and some of them are not amenable to the approximations that serve as guides to the experimental study of the simpler cases. None the less, when occasion demands, the simpler cases can serve as guides to the treatment of the complex.

For the study of a system having even a part of the possible complexity suggested by this compilation, spectrophotometry has one apparent, but not always real, advantage over potentiometry. The latter requires the presence of the oxidized and the reduced components, greatly increasing the number of components; yet electrode measurements provide certain basic data by means of which the free energy changes may be aligned with those of other oxidation-reduction systems. We need not foreshow the importance of this. What may be emphasized now is that each method has distinct limitations.

In order that the part contributed by each reaction to the free energy change of the continuum may be expressed in a manner that helps our understanding of these complex systems, we should have equations relating the experimentally determinable quantities.

A general equation would be unwieldy were it to describe the electrode potential as a function of the activities of all the components that are implied in this list of reactions. Therefore we had best break down the continuum of actually integrated processes and describe the parts by a plexus of equations. Happily, the control of hydrogen ion activities by means of buffer solutions permits us to eliminate consideration of the part played by hydrogen ion activity while developing other features and to deal

with the "pH effect" separately.   Type equations for acid-base equilibria need no repetition.

Equations for Association

There remain for particular consideration the equilibria involving the associations between a nitrogenous base and a metalloporphyrin in the reduced and oxidized states.   These are unique. Equations for a type case were first developed by Clark, Taylor, Davies, and Lewis (15) from the elementary equation of Conant and Tongberg (2).   Only so much of the development will be given here as is necessary to outline the subject and to provide those typical equations that guide experiment.   More detail will be found in the dissertations of Taylor (8), Davies (9), and Vestling (10).

Assume a metalloporphyrin that is subjected to oxidation-reduction in the sense

$$O_n + ne \rightleftharpoons pR_m$$
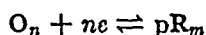
where O_n represents the oxidant in the fixed state of aggregation designated by n, which is also the number of electrons, e, required for the reduction, and R_m represents the reductant in the fixed state of aggregation designated by m.   If n be greater than 1, we shall now assume no intermediate state of reduction, although it will be important to reconsider this limitation should evidence make it necessary.

$$pm = n \qquad\qquad (1)$$

Let both species be under experimental conditions developing no fact that demands consideration of their acid ionizations or ionic charges, as would be necessary to "balance" the "equation" on one side of which appear n electrons.   The electrode equation involving accepted conventions[1] is Equation 2.

$$E_h = E_0 + \frac{RT}{nF} \ln \frac{(O_n)}{(R_m)^p} \qquad\qquad (2)$$

Let a nitrogenous base, B, be added to the solution and there coordinate independently with the oxidized and the reduced

[1] See Clark (16).

metalloporphyrin to form the compounds defined by the following relations.

$$O_n + qB \rightleftharpoons O_n B_q$$

$$R_m + rB \rightleftharpoons R_m B_r$$
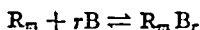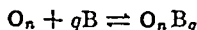
With brackets to indicate molar concentrations, parentheses to indicate activities, and γ to indicate activity coefficients we may state the equilibrium equations in two forms:

$$\frac{(O_n)(B)^q}{(O_n B_q)} = K_O \tag{3, a}$$

$$\frac{[O_n][B]^q}{[O_n B_q]} = \frac{\gamma_{O_n B_q}}{\lambda_{O_n} \gamma_B q} K_O = K'_O \tag{3, b}$$

$$\frac{(R_m)(B)^r}{(R_m B_r)} = K_R \tag{4, a}$$

$$\frac{[R_m][B]^r}{[R_m B_r]} = \frac{\gamma_{R_m B_r}}{\gamma_{R_m} \gamma_B r} K_R = K'_R \tag{4, b}$$

An important assumption is that there is no change in the state of aggregation when metalloporphyrin is converted to base metalloporphyrin.

The assumptions noted will deserve strict attention when the precision of measurement and control of all factors reach artistic adjustment to theoretical requirements. Some will receive further, qualitative treatment later.

Define the following sums

$$S_o = n[O_n] + n[O_n B_q] \tag{5}$$

$$S_r = m[R_m] + m[R_m B_r] \tag{6}$$

With the equations at hand derive Equation 7, a.

$$E_h = E_0 + \frac{RT}{nF} \ln \frac{S_o}{S_r^p} + \frac{RT}{nF} \ln \frac{m^p}{n} + \frac{RT}{nF} \ln \frac{K'_O}{(K'_R)^p}$$

$$+ \frac{RT}{nF} \ln \frac{(K'_R + [B]^r)^p}{K'_O + [B]^q} + \frac{RT}{nF} \ln \frac{\gamma_{O_n}}{\gamma_{R_m^p}} \tag{7, a}$$

If the conditions are such as to permit no serious change in the values of the activity coefficients occurring in Equations 3, b

and 4, b, we may proceed as if the last term in Equation 7, a were constant and K'_o and K'_R were constant.   To simplify the typography we shall omit primes of K'_o and K'_R hereafter.

Proposition I—When all conditions are constant except the ratio of S_o to $(S_r)^p$, symmetry of the titration curve is presumptive evidence that $p = 1$.   See also Proposition XII, b.

Proposition II—If $p = 1$, the slope of the titration curve gives the value of n.

Practically this is best determined by Proposition XVI.   If $p = 1$, $m = n$.   If it be found that $p = m = n = 1$ when sufficient base is present to convert the oxidized and reduced metalloporphyrins practically completely to the respective base metalloporphyrins, it will not necessarily hold that $p = m = n = 1$ for the metalloporphyrin system itself.

We shall continue with the assumption that $p = n = m = 1$ and equal activity coefficients.   These assumptions reduce Equation 7, a to 7, b.

$$E_h = E_0 + 0.0601 \log \frac{S_o}{S_r} + 0.0601 \log \frac{K_o}{K_R} + 0.0601 \log \frac{K_R + [B]^r}{K_o + [B]^q} \quad (7, b)$$

There is included the condition that the temperature is 30°, since the consequences of the numerical coefficient are to be shown graphically.

If [B] be held practically constant, we have Equation 8, to be

$$E_h = E_b + 0.0601 \log \frac{S_o}{S_r} \quad (8)$$

tested by Propositions I and II.   Here E_b indicates the potential that would be observed at 50 per cent reduction of the system at constant concentration of free base, which in most instances will obtain practically at constant concentration of total base.   If no base be present, $E_b = E'_0$.

Proposition III—If the potential increase with addition of coordinating substance, all other conditions remaining constant, $K_o > K_R$.   If the potential change is in the opposite direction, $K_R > K_o$.

We shall confine illustration to the first case.

Proposition IV—If on addition of base, all other conditions remaining constant, the potential approaches a limiting potential, E_2 (see Equation 11), it follows that $q = r$.

The difference $E_2 - E_0$ is determined by the ratio, K_O/K_R, as shown by Equation 11 and as illustrated in Fig. 1. The course of the association curve between E_0 and E_2 is determined by the absolute values of K_O and K_R, and by the concentration of total metalloporphyrin, S.

Particular emphasis may be given to one conclusion that flows from a consideration of these systems and that is implicit in the equations. This is that the change of potential, accompanying the addition of a coordinating base to a fixed mixture of the reduced and oxidized species of the metalloporphyrin, is a measure of the *ratio* of the two dissociation constants. This difference betokens a difference in the free energies of association and where this is large it may need to be considered in the ultimate analysis of the part that complexes of the metalloporphyrins play in catalysis. Furthermore, the fact that other catalysts found in living cells depend upon the association between dissociable prosthetic groups and specific proteins suggests that base metalloporphyrin systems may serve in some degree as models of important general relations. Barron (4) has already pointed to the fact that the potentials of systems containing the same metalloporphyrin can vary considerably with the nature of the base. These specific effects of coordinating bases are referable to the specific values of K_O and K_R. Barron and Hastings (3) and Barron, DeMeio, and Klemperer (17) have applied the shift of potential to the modification of catalytic action.

It is desired to develop Equation 7, b to include the concentration of total coordinating base, S_b, which is directly determinable. The concentration of total base is defined by Equation 9. Other definitions and specifications convenient to the development follow.

$$S_b = [B] + q[OB_q] + r[RB_r] \tag{9}$$

$$S = S_o + S_r \tag{10}$$

$$E_2 = E_0 + 0.0601 \log \frac{K_O}{K_R} \tag{11}$$

$$x = \frac{[O]}{[R]} = \log^{-1} \frac{(E_h - E_0)}{0.0601} \tag{12}$$

$$y = \frac{[OB_q]}{[RB_r]} = \log^{-1} \frac{(E_h - E_2)}{0.0601} \tag{13}$$

$$\alpha = \frac{S_0}{S} \tag{14}$$

$$\beta = \frac{[OB_q] + [RB_r]}{S} \tag{15}$$

With these relations Equation 16 may be derived.

$$E_h = E_0 + 0.0601 \log \frac{S_o}{S_r} + 0.0601 \log \frac{K_o}{K_R} + 0.0601 \log \frac{K_R + (S_b - ZS)^r}{K_o + (S_b - ZS)^q} \tag{16}$$

where, for the restriction that $q = r$

$$Z = \frac{r(y + 1)}{(x - y)} [x(1 - \alpha) - \alpha] \tag{17}$$

and for the further restrictions that $\alpha = 0.5$ and $r = 2$

$$Z = \frac{(y + 1)(x - 1)}{(x - y)} \tag{18}$$

For the restriction that $\alpha = 0.5$ but no limitation on the relation of q to r

$$Z = \frac{(qy + r)(x - 1)}{(x - y)} \tag{19}$$

Proceed, for the moment, with Equations 16 and 18.

Proposition V—Certain first order, graphical approximations may give useful information, as detailed below.

In the case selected, unique points of the association curve are (*a*) where

$$[B]^2 = K_R \text{ or } 2 \log [B] = \log K_R \tag{20}$$

There $Z = 0.5$, $E_h - E_0 \fallingdotseq 0.0181$, $S_b \fallingdotseq K_R - 0.5S$. The last two relations and the known values of S_b and S make it possible to judge when $S_b \fallingdotseq [B]$. This approximation is least valid at the low parts of the association curve, but, especially when the value of S is low, it will be found frequently that it may be used profit-

ably if not extended below this first unique point. Then S_b may replace [B] in Equation 7, b and modified Equation 7, b may be used in place of the unwieldy Equation 16; (b) the center point of inflection where

$$[B]^4 = K_0 K_R \qquad (21)$$

There $Z = 1$, $[B] = (S_b - S)$, $\beta = 0.5$, $[RB_r] = [O]$, $[R] = [OB_q]$, and $E_h = (E_0 + E_2)/2$; (c) where

$$[B]^2 = K_0 \text{ or } 2 \log [B] = \log K_0 \qquad (22)$$

There $Z = 1.5$ and $E_2 - E_h = 0.0181$.
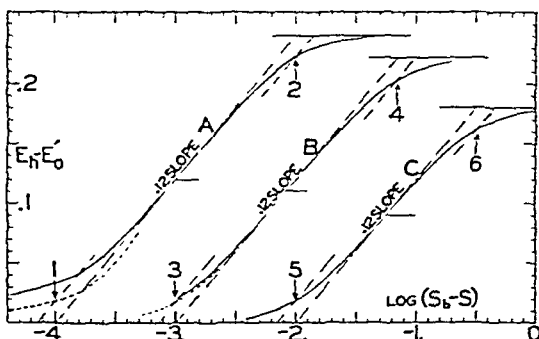
FIG. 1. The solid lines represent the theoretical relation of $E_h - E'_0$ to $\log (S_b - S)$ when $q = r = 2$, $0.5S = S_r = S_0 = 1 \times 10^{-4}$ M; the broken lines, the same with $\log (S_b - 0.5S)$ as abscissa. Curve A, $K_R = 1 \times 10^{-8}$, $0.5 \log K_R = -4.00$ (see Point 1); $K_0 = 1 \times 10^{-4}$, $0.5 \log K_0 = -2.00$ (see Point 2); $E_2 - E_0 = 0.2400$ (0.06 coefficient). Curve B, $K_R = 1 \times 10^{-6}$, $0.5 \log K_R = -3.00$ (see Point 3); $K_0 = 5 \times 10^{-3}$, $0.5 \log K_0 = -1.15$ (see Point 4); $E_2 - E_0 = 0.2219$ (0.06 coefficient). Curve C, $K_R = 1 \times 10^{-4}$, $0.5 \log K_R = -2.00$ (see Point 5); $K_0 = 1 \times 10^{-1}$, $0.5 \log K_0 = -0.50$ (see Point 6); $E_2 - E_0 = 0.1800$ (0.06 coefficient).

For the case under consideration it may be shown that, when $K_0 > S_b{}^r > K_R$ at the center point, the slope at the mid-point approaches the uniquely simple, algebraic form (Equation 23):

$$\frac{dE}{d \log(S_b - S)} \doteq (0.0601)r \qquad (23)$$

Fig. 1 illustrates a method of graphical analysis based upon the use of these approximations and unique points. Since Z is

unity at the center point, log $(S_b - S)$ is made the abscissa in order that the important orienting diagonal, of slope 0.12, may coincide with the center point.   When the ratio of K_O to K_R is as large as any of the values indicated, the diagonal is very close to the tangent through the mid-point and accordingly can be well placed.   This diagonal will intersect the line of E'_0 where

$$0.5 \log K_R = \log [B] \tag{24}$$

Now if $[B] \doteqdot (S_b - S)$, as it does in Curve C, this point of intersection will be 0.0181 volt below the corresponding point of the association curve, drawn with log $(S_b - S)$ as abscissa.   Curves B and A show progressively greater departure from this relation. Yet at the unique point in question $Z = 0.5$, so that, if a supplementary curve be plotted with log $(S_b - 0.5S)$ as abscissa, the unique point falls on the supplementary curve very close to 0.0181 volt above the aforementioned intersection.   We shall show that it is not always possible to report a reliable value for E'_0.   When deprived of this base-line, one may use the obvious, alternative method shown in Fig. 1; namely, to intersect the appropriate curve with a line parallel to the orienting diagonal and 0.0181 volt above. For the curves shown and on the scale used in Fig. 1 it would be difficult to discern any difference along the upper part of the curves whether the abscissa were made log $(S_b - S)$, log S_b, or the log $(S_b - 1.5S)$ which is demanded for the location of the unique point where

$$0.5 \log K_O = \log [B] \tag{25}$$

If necessary a drawing of larger scale may be used and the principle described for the finding of K_R applied to the estimation of K_O.   Fig. 1 illustrates the elements.

Once the orders of magnitude of K_O and K_R, as well as of S, are known, one may estimate the legitimacy of each approximation.   In extreme cases it may be necessary to resort to a series of progressively closer approximations but, especially when S is small and the constants large, it suffices to plot with log S_b. The latter is preferable for final description.

It should not go unnoted that previous authors have plotted with S_b as abscissa.   The resulting graph obscures those features that are useful in the estimation of K_R and K_O and it may leave

the impression that the potentials at high concentration of base have reached a limit when the evidence thereof is quite inadequate.

Proposition VI—If at and near the center of an association curve K_O is much greater than $[B]^q$ and K_R is much less than $[B]^r$, the value of r will be determined by the maximal slope of the association curve, which is given by Equation 23.

The actual slope need only approximate an integral multiple of 0.0601, since r must be a whole number. The departure will be the greater the smaller the ratio of K_O to K_R. If q be less than r, the effect of combination between base and oxidant will extend over a larger range and may affect the slope of the midsection.

Comment on Stepwise Association—If association be stepwise, a term such as $K_O + [B]^2$ in Equation 7, b must be replaced by

$$K_{O_1}K_{O_2} + K_{O_1}[B] + [B]^2 \tag{26}$$

or by a more extended term if the number of steps be greater than two. On statistical grounds stepwise association is more probable than concurrent addition of 2 or more molecules of base and under certain distributions of the energy changes this may be reflected in the equilibrium states. Therefore the theorist will be tempted to provide for this. On the other hand the experimentalist will recognize that the provision of several constants in terms of this kind is not unlike the provision of several constants in those infinite series that are used for the development of empirical equations and becomes devoid of physical significance if his method be not delicate enough to detect the individual values. But be it noted that, as $[B]$ varies, the value of the sum in Equation 26 runs from that of the first term to that of the last. Accordingly, for the purpose of defining the gross contour of an association curve, Equation 26 may be reduced to the corresponding term in Equation 7, b.

The further the departure between the values of the first and second dissociation constants, the more will be the weight of a $K_1[B]$ term, so that experimental identification may become possible. When it is, the equations may be enlarged appropriately.

A Note on Polymers—The potentiometric method can reveal

the presence of polymers. A particular case was described by Stichler and Clark (18). Barron (4) and Davies (9) show that the components of certain metalloporphyrin systems are dimers. The equations here given are predicated on the assumption that there is no *change* in state of aggregation and where the symbols that have been used imply monomeric components the equations need only slight modification to represent the case in which all metalloporphyrins are dimeric. The situation is entirely different when there is a *change* in state of aggregation accompanying association with a base. Because of the much greater complexity
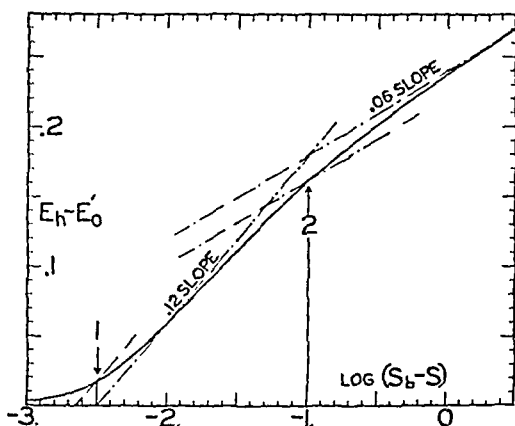
FIG. 2. Theoretical curve. Case of $q = 1$, $r = 2$, $K_R = 1 \times 10^{-5}$, $K_0 = 1 \times 10^{-1}$. Point 1, $0.5 \log K_R = \log (S_b - S)$; Point 2, $\log K_0 = \log (S_b - S)$.

of such equations we think it wise to postpone their treatment until necessity demands it.

Cases in Which $q < r$ —For such cases Propositions I, II, III, and VI will hold, while IV and V need modification. Fig. 2 illustrates a theoretical case and a method of graphical analysis so similar to that already described as to require no further comment. Fig. 2 will also serve to illustrate some of the propositions that follow.

Proposition VII—If $q < r$, the maintenance of the slope r (0.0601) at high values of [B] should indicate that K_0 is infinite, and $q = 0$ (no association of oxidized metalloporphyrin and base).

Proposition VIII—A decline of the slope on further addition of base indicates that K_0 is finite and $q > 0$.

On still further addition of base the term $0.0601 \log (K_E + [B]^r)/(K_0 + [B]^q)$ approaches $0.0601 \log [B]^r/[B]^q$ or $0.0601 \log (S_b)^{r-q}$ (see Fig. 2). Whence:

Proposition IX—If, at very high concentration of coordinating base, the slope of the association curve approaches a constant value, the slope is given by Equation 27 from which is determined the value of $r - q$.

$$\frac{\Delta E_h}{\Delta \log S_b} = 0.0601(r - q) \tag{27}$$

Participation of Hydrions—To avoid unnecessary complexity we have followed the policy of breaking down the continuum of actually integrated processes and up to this point have left out of consideration the participation of hydrogen (or hydroxyl) ions. That this is a practical policy flows from the established fact that, if a set of measurements be made in buffer solutions at constant pH, the components of the buffer system will absorb or supply the hydrogen ions liberated or taken up in the reactions hitherto considered and no experimental evidence of their participation will appear, provided, of course, that the buffer system be in great excess. By the same token, a shift in the pH value of the buffer system will alter the ease with which hydrogen ions are made available for the completion of the reaction and the effect will appear in the magnitude of the over-all free energy change, the intensity factor of which is measured by the electrode method.

Since these principles have been developed in previous studies (19), it seems unnecessary to embody them in equations now being used to outline general features, although it will be essential to do so in dealing with specific, actual cases. But there is one aspect that should be given particular emphasis lest the policy now being followed become grievously deceptive.

Prior to potentiometric studies by Conant (1, 2) and Barron (4) it was largely a matter of artistry to assign to ferriprotoporphyrin in alkaline solution a hydroxyl ion, or a species formula that we shall represent by $O\overline{OH}$. Now that potentiometric measurements have shown hydrogen ions or hydroxyl ions to be involved in the equilibria obtaining among some of the systems, we must contemplate the possibility that there is a competition between hydroxyl ions and coordinating base molecules for posi-

tion at the coordination center. To take care of this in the simplest of several possible formulations let us postulate that in the coordination between base and oxidized metalloporphyrin the base coordinates only with the species O, leaving applicable Equation 3, b but that, in addition, there obtains the equilibrium represented in Equation 3, c.

$$\frac{[OOH][H^+]}{[O]} = k[H_2O] = K_h \qquad (3,\ c)$$

By following the usual procedure with the inclusion of Equation 3, c one arrives at an "electrode equation" comparable with Equation 7, b and differing therefrom only in the last term. This term when written in a form suited to the immediate discussion is

$$0.0601 \log \frac{K_R + [B]^r}{\dfrac{K_o}{[H^+]}([H^+] + K_h) + [B]^q} \qquad (28)$$

The point to be emphasized is that when association experiments are run at *constant* hydrogen ion concentration the constant called K_o in the foregoing discussion should be in reality $(K_o/[H^+])([H^+] + K_h)$. In other words what has been called the dissociation constant of the base metalloporphyrin in oxidized form is an "apparent constant" the value of which is a function of $[H^+]$.

The general significance of this warning is not dependent upon the particular postulate used above—a postulate which we shall show is too simple.

Proposition X—Previous theoretical and experimental studies (19, 20) have shown that, when the relation between E_h and pH, other conditions being constant, is

$$\frac{-\Delta E_h}{\Delta\ pH} = 0.0601 \text{ (at } 30°) \qquad (29)$$

the reductant must possess 1 more hydrogen ion (or 1 less hydroxyl ion) than the oxidant for each electron necessary to convert oxidant to reductant.

Evidence will be presented in the experimental papers that the pH effects have to do with the coordination shell so that we may now say that the above relation will demonstrate the reduced

coordination center to possess 1 more hydrogen ion or 1 less hydroxyl ion per metal atom.

Proposition XI—On increase of pH, if the *reductant* lose a equivalents of protons per mole, the value of $-dE_h/d$ pH will decrease by $a(0.0601)/n$ (at 30°); while if the *oxidant* lose a equivalents of protons per mole the value of $-dE_h/d$ pH will increase by $a(0.0601)/n$. Loss of a proton is equivalent to gain of hydroxyl ion. See references (19) and (20) for details on this proposition.

An example will be shown of a change from $-\Delta E_h/\Delta$ pH $=$ 0.0601, for the higher values of pH, to $-\Delta E_h/\Delta$ pH $= 0.0$ for the lower values of pH. Whatever the structural interpretation may be, this experimental fact demands the *equivalent* of the loss of hydroxyl ion from the oxidant at the lower pH values. Also the mid-point of the transition between the two slopes gives the so called pK value.

Comment on Activities—There remain for consideration troublesome questions arising from changes in activity coefficients that have been neglected up to this point. A rigid treatment of this subject, involving so complicated a set of components, would require a length out of proportion to the scope of the experiments to be reported. Therefore, we may reduce our considerations to the following brief summary of our policy regarding the least certain of the several experimental aspects of this subject.

Simultaneous control of all factors but the one under immediate investigation is not rigidly attained in actual experiments. The greater uncertainties lie in the interpretation of data purporting to yield "pH" values and in corrections for those changes of pH and other factors that attend the modification of the medium. To provide a basis for the discussion of the first it seems advisable to outline here the experimental procedure.

pH Control—In many cases the concentration of added base, such as pyridine or nicotine, is so large as to make impracticable the use of the pH numbers of the solutions containing only the buffer salts. Furthermore, such substances interfere with the proper operation of the hydrogen electrode in the solutions used. Accordingly the glass electrode was employed. The type introduced by MacInnes and Belcher (21) was operated in a shielded, constant temperature, air bath. A Compton quadrant electrome-

ter was used for null point instrument in conjunction with a Leeds and Northrup type K potentiometer.   A very close approach to the theoretical change of potential with pH was established with acid and neutral buffers that had been measured with the hydrogen electrode.   The glass membrane had to be used with alkaline solutions with which there are well known difficulties.   The use of potassium in place of sodium in the buffers materially reduced drifts and deviations.   Since the membrane of Corning No. 015 glass behaved normally with mildly acidic buffers, if previous contact with alkaline buffers had not been prolonged, it was assumed that its initial response in alkaline solution was the more reliable.   Accordingly rapid measurements were made of the tested solution and of buffer solutions of nearly the same pH numbers, as determined by the hydrogen electrode, and interpolations were made.   By this procedure there were found orderly relations between the potentials of the cell with glass membrane and the amounts of alkali added to any one of the various buffer solutions and also orderly relations between the potentials and the amounts of pyridine or alcohol added to aqueous buffers.

If we accept a potential so measured as indicative of a pH number, that number appears to be referred to the standard for aqueous solutions and as if measured with the hydrogen electrode, since the latter remained the means of standardizing the compared solutions.   Actually the higher concentrations of pyridine, or nicotine, which in some cases went as high as 3 M, may seriously impair the ordinary interpretation of "pH" and of the standard methods of its computation.   Nevertheless, if any one set of data be restricted to solutions having grossly the same composition and if it then be found, on making a "pH" change the major variable, that there is established a close approach to the pH effect predicted by the simplified equations, the following conclusion is drawn.   While strict standardization of pH numbers is precluded, the experimental satisfaction of the predicted pH effect in terms of *relative* pH changes establishes the *nature* of a part of the equilibrium.   This, rather than precisely referred values, we consider to be of first order importance.

The greater uncertainties are in the experiments purporting to show the course of association.   Here occur the largest of the uncontrolled changes of the medium.   What we have been forced

to assume are the following. (*a*) The shift of pH accompanying the addition of neutral pyridine, nicotine, etc., is given by glass electrode measurements and can be applied by use of the established pH effect so as to bring all points on the association curve to the basis of constant "pH" on the arbitrary scale. (*b*) The shift of the activity coefficients is *assumed* to be negligible while coordinating base is increased. Here we may retain the same order of magnitude of the ionic strength but the dielectric constant will change.

APPLICATIONS OF SPECTROPHOTOMETRY[2]

Granting that obvious prerequisites for spectrophotometry are met and that *only two* species containing metalloporphyrin absorb at a suitably chosen wave-length, we may use the following relations.

Assume the process

$$aM_n + bA \rightleftharpoons cM_n A_e \tag{30}$$

where A represents either (*a*) base coordinating with M_n at constant pH to form $M_n A_e$ or (*b*) hydrion adding to M_n to form $M_n A_e$ at constant concentration of base. In case (*b*), M may be either metalloporphyrin or base metalloporphyrin. Relation 30 is stated in this general form for economy of space and to avoid needless repetition for the several specific cases. The restriction to two absorbing species places a severe limitation upon the following relations. Relief from this restriction will be noted in a special, experimental case.

Implied in Relation 30 are that:

$$am = cn \tag{31}$$

$$b = ce \tag{32}$$

$$\frac{[M_m]^a [A]^b}{[M_n A_e]^c} = K \tag{33}$$

Define

$$S = m[M_m] + n[M_n A_e] \tag{34}$$

$$\epsilon' = -\log T \tag{35}$$

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[2] See also Hogness *et al.* (5).

where T is the transmittance for any mixture of absorbing species at a fixed value of S and given length of absorbing column.[3]

$$\epsilon'_1 = -\log T_{Mm} \tag{36}$$

where T_{Mm} is the limiting value of T where $S = m[M_m]$.

$$\epsilon'_2 = -\log T_{MnAe} \tag{37}$$

where T_{MnAe} is the limiting value of T when $S = n[M_nA_e]$. By application of the rule of the additive property of log transmittances one obtains

$$\frac{[M_m]^a}{[M_nA_e]^c} = \frac{K}{[A]^b} = S^{a-c}n^c m^{-a}(\epsilon'_1 - \epsilon'_2)^{c-a}\frac{(\epsilon'_2 - \epsilon')^a}{(\epsilon' - \epsilon'_1)^c} \tag{38}$$

Proposition XII—(a) Symmetry of the curve relating ϵ' to log $[A]$ is presumptive evidence that $a = c$, whence $m = n$.

(b) Absence of a "dilution effect" involving S^{a-c} is further evidence. When $a = c$ and $m = n$, Equation 38 reduces to Equation 41 with appropriate interpretations of b and K.

If S is constant, the tangent to the curve at the mid-point, where $\epsilon' - \epsilon'_2 = \epsilon'_1 - \epsilon'$, will be given by

$$\frac{d \log [A]}{d\epsilon'} = \frac{-0.8686}{(\epsilon'_1 - \epsilon'_2)}\left(\frac{a + c}{b}\right) \tag{39}$$

Practically the use of this tangent may not be very certain, but to illustrate uses, consider the following possible cases.

Case 1. $M\ + 2A \rightleftharpoons MA_2$; $a = 1, b = 2, c = 1$
"   2. $2M + 3A \rightleftharpoons M_2A_3$; " $= 2,$ " $= 3,$ " $= 1$
"   3. $M_2 + 2A \rightleftharpoons M_2A_2$; " $= 1,$ " $= 2,$ " $= 1$

All of these give the same value of $(a + c)/b$. Yet Cases 2 and 3 would require 2 equivalents for the oxidation-reduction of the *base* metalloporphyrin. Therefore, if a symmetrical, potentiometric curve involving 1 equivalent were to be found, Case 1 could be presumed to apply and the slope now under discussion would indicate that $b = 2$. An additional and better test for

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[3] The symbol ϵ' is introduced for economy of space in the more complicated equations. With apologies to the reader we ask that he carefully distinguish ϵ' from ϵ (see Table I).

the value of b is discussed in the following section. Be it noted that the spectrophotometric test alone would not distinguish Case 1 from Case 3.

Proposition XIII—If $a = c$ the center of the symmetrical curve relating ϵ' to log $[A]$ is where $\epsilon'_2 - \epsilon' = \epsilon' - \epsilon'_1$ and at this point

$$\log K = b \log [A] \tag{40}$$

RECTIFICATION OF CURVES

The general method of Reed and Berkson (22) is used.

Rectification of Spectrophotometric Curves. Relation of $- Log$ T (or ϵ') to Log A—This is limited to the following modification of Equation 38.

$$\frac{[M_m]}{[M_m A_b]} = \frac{K}{[A]^b} = \frac{\epsilon'_2 - \epsilon'}{\epsilon' - \epsilon'_1} \tag{41}$$

Choose an orienting pair of values called ϵ'_0 and log $[A]_0$ and define

$$b \log [A] - b \log [A]_0 = p \tag{42}$$

$$\log K - b \log [A]_0 = -\log G \tag{43}$$

where $p = 0$, when $\epsilon' = \epsilon'_0$. Derive

$$G\, 10^p = \frac{\epsilon' - \epsilon'_1}{\epsilon'_2 - \epsilon'} \tag{44}$$

$$G = \frac{\epsilon'_0 - \epsilon'_1}{\epsilon'_2 - \epsilon'_0} \tag{45}$$

Whence

$$\epsilon' = \epsilon'_1 + (G + 1) \frac{(\epsilon'_0 - \epsilon')10^p}{1 - 10^p} \tag{46}$$

Proposition XIV—If the assumed value of b fits the actual case, a chart of ϵ' against $((\epsilon'_0 - \epsilon')10^p)/(1 - 10^p)$ should give a straight line the properties of which determine K, ϵ'_1, and ϵ'_2.

The slope is $(G + 1)$, whence K is found by Equation 43. The intersection with the ϵ' axis gives ϵ'_1. Thence ϵ'_2 is found by Equation 45. If desired, the equation can be recast to yield ϵ'_2 by intersection.

Rectification of Potentiometric Association Curves Relating E_h *to* *Log* S_b—Let Equation 7, b be solved for $[B]^q$ under the condition that $q = r$. Assume $[B] \fallingdotseq S_b$. Define

$$\log^{-1} \frac{E_h}{0.0601} = u \tag{47}$$

$$\log^{-1} \frac{E_2}{0.0601} = J \tag{48}$$

$$q \log S_b - q \log S_{bo} = p \tag{49}$$

$$-q \log S_{bo} = \log C \tag{50}$$

The chosen pair of orienting data are u_o and S_{bo} where $p = 0$. Derive

$$u = J + (CK_O + 1) \frac{u_o - u}{10^p - 1} \tag{51}$$

Proposition XV—When previous knowledge justifies the use of the approximation carried into Equation 51, the slope of the straight line relating u to $(u_o - u)/(10^p - 1)$ is $(CK_O + 1)$ which yields the value of K_O by use of Equation 50 and the intersection with the u axis yields E_2 by Equation 48.

Similar relations leading to the evaluation of K_R and E'_0 could be obtained by use of $u' = \log^{-1} (-E_h/0.0601)$ and $J' = \log^{-1} (-E_0/0.0601)$.

The use of this method of rectification is tricky for the following reasons.

If the orienting values of u_o and $\log S_{bo}$ be taken from near the center of the association curve where the effects of K_O and K_R are small, the slope of the rectified line approaches unity and is of no value in the determination of either constant. The approximation carried into the equation, namely $S_b \fallingdotseq [B]$, is least valid at the lower part of the association curve. Therefore we hesitate to use this method for evaluating K_R. The following simpler rectification may be applicable in rare cases. Assume $q = r$, $S_o = S_r$, $S_b = [B]$, $K_O >> [B]^q$. Whence $E_h = E'_0 + 0.0601 \log (K_R + S_b^r)/K_R$. Define $u = \log^{-1} (E_h/0.0601)$ and $E_0 = 0.0601 \log W$. Whence $u = W + (W/K_R) S_b^r$. Thus a plot of u against S_b^r should approximate a straight line the proper-

ties of which allow estimate of K_R but only a crude estimate of E'_0.

Rectification of Titration Curves

Reed and Berkson's method as applied to this case has been discussed by Clark and Perkins (23). We shall use the symbols defined by the latter authors as follows:

$y \equiv$ number of ml. of specified reducing agent required to reach the potential E_h

$d \equiv$ number of ml. of the reducing agent added before the main system is attacked

$A \equiv$ number of ml. of the reducing agent required to carry the main component from 0 to 100 per cent reduction

E_n and y_n are chosen orienting values of E_h and y respectively

$$P = \frac{E_h + E_n}{1/n(0.0601)} \tag{52}$$

$$E'_0 + E_n = -\frac{0.0601}{n}\log C \tag{53}$$

Whence

$$y = (C + 1)\frac{y_n + y10^P}{1 - 10^P} - dC \tag{54}$$

Proposition XVI—The properties of a titration curve are determined by the properties of a straight line the coordinates of which are y and $(y_n + y10^P)/(1 - 10^P)$ according to Equation 54.

The mid-point potential, E'_0, is determined by the slope $(C + 1)$ and thence all other characteristics of the titration curve are evaluated as described by Clark and Perkins.

The method is predicated on the applicability of Equation 8 and in particular upon an integral value of n. It is important to note the following. It is possible that the method may yield such numerical values of the constants that a theoretical, sigmoid curve, constructed with these values, may be shifted to a position of best correspondence with an actual titration curve that is slightly asymmetric. To be sure, only a slight asymmetry would go undetected; nevertheless there remains danger of overlooking something. For the present we may say that we know

of no better criterion of the validity of deduced values to be reported unless it be so extreme a precision of measurement and so rigid a control of variables as to make the mathematical analysis by the method of Reed and Berkson superfluous. All titration data are analyzed by this method.

SUMMARY

A nomenclature for metalloporphyrins and their coordination compounds is proposed.

The reactions that may take place in systems containing metalloporphyrins and coordinating bases are typed to define the restrictions of the immediate study.

There are developed theoretical equations relating the electrode potential, the concentration of total metalloporphyrin, the total base that coordinates independently with reduced and oxidized metalloporphyrin, and the ratio of concentrations of oxidized and reduced metalloporphyrin.

A brief review is given of the principles governing the study of the part played by the acid-base equilibria and of experimental difficulties in the application of these principles to the cases at hand.

Approximate forms of the equations are developed to show graphical methods of determining the constants that are descriptive of the dissociation of oxidized and reduced coordination complexes and to show methods of determining the numbers of molecules of coordinating substance that associate on the one hand with reduced metalloporphyrin and on the other hand with oxidized metalloporphyrin.

Equations are given for the use of spectrophotometric data in determining the various constants that define the equilibria of the systems.

Equations are given for rectification of curves.

Sixteen propositions and corollaries thereto are offered as subject to the experimental tests applied in the subsequent papers.

$[B]$ = molar concentration of free, uncombined, coordinating base

E_λ = electrode potential referred to the hydrogen standard

$E_b = E_\lambda$ at constant S_b, other conditions being specified in the text

$E'_0 = E_\lambda$ at 50% reduction of metalloporphyrin, $S_b = 0$, other conditions being specified in the text

e = 1 equivalent of electrons

F = the faraday

K_O = dissociation constant of oxidized base metalloporphyrin

K_R = dissociation constant of reduced base metalloporphyrin. Note, special dissociation constants are defined as needed

n = (1) number of equivalents required to reduce 1 mole of oxidant; (2) number of gm. atoms of metal per mole of oxidant

$[O_n]$ = molar concentration of oxidized metalloporphyrin containing n gm. atoms of metal ion per mole

$[O\overline{O}H]$ = molar concentration of oxidized metalloporphyrin associated with 1 mole of hydroxyl ions per mole

$[B_qO_n]$ = molar concentration of oxidized, base metalloporphyrin containing q moles of base per mole

$\epsilon' = -\log T = \epsilon cl$

p = ratio of gm. atoms of metal per mole of oxidant to gm. atoms of metal per mole of reductant

q = number of moles of base associating with 1 mole of oxidized metalloporphyrin

r = number of moles of base associating with 1 mole of reduced metalloporphyrin

$[R_m]$ = molar concentration of reduced metalloporphyrin

$[B_rR_m]$ = molar concentration of base metalloporphyrin containing r moles of base per mole

R = gas constant

$S = S_o + S_r$

S_o = sum of apparent molar concentrations of oxidized metalloporphyrin and oxidized base metalloporphyrins

S_r = sum of apparent molar concentration of reduced metalloporphyrin and reduced base metalloporphyrins

S_b = sum of apparent molar concentrations of uncombined base and base metalloporphyrins

T = absolute temperature

T = transmittance

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METALLOPORPHYRINS

II. COBALT AND MANGANESE MESOPORPHYRINS IN COORDINATION WITH NITROGENOUS BASES*

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The introduction to this series of reports (1) has indicated that we may expect to elucidate clearly the main features of systems containing metalloporphyrins and nitrogenous bases that coordinate therewith only when properties pertaining to each of the several components are varied independently. Accordingly, metal, porphyrin, and base, as well as the hydrogen ion activity and the ionic strength of the solutions, have been varied in a series of measurements of the equilibria obtaining among these components.

To initiate such studies in this laboratory metallo compounds of mesoporphyrin IX were chosen because this porphyrin is somewhat more stable than protoporphyrin. Protoporphyrin IX is the porphyrin component of those iron porphyrins previously studied by Conant and his students (2) and by Barron (3). We were surprised to find that the hydrogenation of the vinyl groups of protoporphyrin was accompanied by certain undesirable changes in the properties of the corresponding metalloporphyrins. In particular the solubilities of reduced metalloporphyrins formed from mesoporphyrin are low and this has seriously limited the range of our experiments.

Laidlaw (4), who first prepared a cobalt porphyrin, recognized that it could be oxidized and reduced. Zaleski (5) prepared manganese mesoporphyrin, which Barcroft (6) mentions can be

oxidized and reduced.   It is our purpose to show that manganese and cobalt mesoporphyrins, as do metalloporphyrins which contain iron, participate in several of the equilibria previously outlined (1), more particularly in those of oxidation-reduction and association with a base.

Preparations

Hemin chloride, ferriprotoporphyrin IX chloride, was prepared from washed pig erythrocytes by a slight modification of the method of Schalfejew (7), improved by Willstätter and Fischer (8). It was thrice recrystallized by the quinine-chloroform method as suggested to us by Barron (see (9)).

Mesoporphyrin IX hydrochloride can be prepared from hemin chloride by simultaneous reduction and removal of iron.   Three different methods gave indistinguishable products.   The method most satisfactory in our hands was the following.   Hemin chloride (3 gm.) is finely ground and suspended in formic acid (250 ml., 87 per cent).   While the mixture is being heated under a reflux condenser, small portions of palladium catalyst, prepared by Adams' method (10), are added.   The progress of the reaction is followed spectroscopically.   There appears first the spectrum of protoporphyrin as sufficient reduction takes place to form the dissociable ferroprotoporphyrin.   This spectrum gradually gives place to that of mesoporphyrin, and in order that the product may contain minimal quantities of protoporphyrin it is advisable to continue the heating until no trace of the protoporphyrin spectrum can be observed in as concentrated an ethereal solution of the reaction mixture as is compatible with distinguishing the closely placed bands of these two compounds.   Heating should then be discontinued, for it is evident from what appears in the next step that additional reactions, which may be called those of "decomposition," are taking place.   There will be the temptation to continue the heating to increase the yield, since considerable undissolved hemin remains.   Some compromise is advisable. 2 to 3 hours are usually required.   The cooled solution is filtered and poured slowly into 30 per cent ammonium acetate solution (1 liter).   The precipitate is collected by centrifugation and is washed repeatedly with water.   It is taken up in sodium hydroxide solution with great care to use as low a concentration of alkali

as is practicable. The solution is filtered, and the sodium "salt" of mesoporphyrin is "salted-out" with sodium tartrate. The centrifugation of this precipitate reveals the "decomposition" products, for when the material is washed with sodium tartrate solution (2 per cent), the dark, brownish colored supernatant gradually gives place to a clear solution, leaving a bright, red precipitate. It is advisable to repeat the solution in water and salting-out with sodium tartrate, and in doing so it is essential that the precipitate be "cut" sharply from the mother liquor, lest the salt in the latter prevent solution in any but large quantities of water. The sodium salt is converted into mesoporphyrin hydrochloride by throwing upon the precipitate (in the centrifuge cups) boiling 2.5 per cent hydrochloric acid solution (200 ml.). The solid dissolves instantly and the crystals of the hydrochloride form almost immediately, so that if filtration is desired before the crystallization one must operate quickly with an efficient suction filter. The crystals are washed with a little 2.5 per cent hydrochloric acid solution, and dried in air.

Identification—Samples of mesoporphyrin hydrochloride prepared by different methods were compared in the spectrophotometer. The several samples when compared in any *one* of several sorts of solution gave essentially the same absorption curves and molar extinction coefficients.

Portions of each preparation were converted to the dimethyl ester, which was recrystallized from methyl alcohol and chloroform. The "melting points" were observed in capillary tubes, immersed in a vigorously stirred bath of Crisco, electrically heated at various controlled rates. Thus it was possible to approach the expected melting point so steadily and slowly that there was ample time to make the lowest adjustments of the White potentiometer (corresponding to about 0.02°) with which a copper-constantan thermocouple was read. The thermocouple, made of tested wires, was calibrated in boiling water and in boiling naphthalene. None of the samples melted with ideal sharpness. The general picture was that described by White (11) as characteristic of a slightly impure substance. The possibility of some decomposition during the melting in open capillary tubes exposed to air cannot be excluded.

A sample of the dimethyl ester of the mesoporphyrin hydro-

chloride, prepared as described, "melted" at 215.6° and 215.7°, while a sample obtained some years ago from Professor Hans Fischer gave an average "melting point" of 213°. The several values for mesoporphyrin IX dimethyl ester, reported in the course of years from Professor Fischer's laboratory, range from 210° to as high as 216° (Fischer and Stangler (12)). Mixtures of our samples in various proportions with that from Professor Fischer "melted" at 215.1°, 215.0°, and 215.5°.

Manganese mesoporphyrin was prepared by a slight modification of the method of Zaleski (5). Mesoporphyrin hydrochloride (2.5 gm.) was dissolved in hot glacial acetic acid (75 ml.). Manganese acetate tetrahydrate, obtained from de Haën, and reported to contain not over 0.00003 per cent Fe, 0.0007 per cent heavy metals, 0.001 per cent Mg, and 0.0001 per cent Ca, was added as a solution of 5 gm. in 25 ml. of 60 per cent acetic acid. The reaction mixture was heated, with shaking to promote oxidation by air, and presently diluted with 50 ml. of water. Heating was continued until there appeared in the spectrum a band at 460 mμ characteristic of the manganese mesoporphyrin. After the spectral bands of the porphyrin had disappeared completely, the solution was concentrated *in vacuo* to 25 ml. Addition of water to the warm, concentrated solution produced a copious precipitate, which, after being washed with water and dried *in vacuo* over calcium chloride and potassium hydroxide, amounted to 1.96 gm. The material is too soluble in glacial acetic acid to be recrystallized by the quinine-chloroform method, or other methods employed with hemin chloride.

Cobalt Mesoporphyrin—If one treats a glacial acetic acid solution of mesoporphyrin hydrochloride with a solution of cobaltous acetate in the same solvent, one observes absorption bands characteristic of a metalloporphyrin, and the precipitation of fine, red, bent needles. Upon dissolving the precipitate in aqueous alkali one observes the two-banded spectrum of cobaltimesoporphyrin so that one may be misled into the belief that the original preparation is also the oxidized form. Actually it is cobaltomesoporphyrin, which is but slowly oxidized by air under the conditions of its formation, yet is rapidly oxidized in aqueous alkali. The precipitate forms so rapidly under certain circumstances that it occludes considerable mesoporphyrin, easily recognized

by the spectrum in nicotine solution. Fischer, Treibs, and Zeile (13) report similar occlusion in the preparation of protohemin. The best preparations were obtained by delaying precipitation, and operating under purified nitrogen.

Cobaltous acetate was prepared from Merck reagent $Co(NO_3)_2$.--$6H_2O$, "low in nickel," for which the reported limits of impurities were 0.01 per cent Ni, 0.001 per cent Fe, 0.000 per cent Cu, 0.03 per cent Zn, and 0.000 per cent Pb. The carbonate was precipitated by reagent potassium carbonate, washed free of nitrate, taken up in glacial acetic acid, and the solution concentrated *in vacuo* over potassium hydroxide and calcium chloride until the solid phase formed. 1 gm. of this material was dissolved in hot glacial acetic acid (1 liter). Mesoporphyrin hydrochloride (0.5 gm.) was dissolved in another portion of hot glacial acetic acid (0.5 liter). After each solution had been deaerated by a stream of pure nitrogen, the porphyrin solution was added dropwise to the solution of cobaltous acetate, while the latter was stirred mechanically and by the stream of nitrogen. The addition was controlled, spectroscopically, to prevent the accumulation of excess porphyrin. Precipitation began after about one-fourth of the porphyrin had been added. Finally the mixture was cooled under nitrogen, the precipitate was collected rapidly, washed in the filter with cold, oxygen-free glacial acetic acid, and dried *in vacuo* over potassium hydroxide. Yield 0.38 gm.

That the material which separates during the preparation is cobaltomesoporphyrin and that this is oxidized by air when it is brought into aqueous alkaline solution is shown quantitatively as follows: Samples were weighed into the side arms of Warburg manometer vessels, in the main chambers of which was placed aqueous alkaline solution of nicotine sufficient to dissolve the cobalt mesoporphyrin. After the vessels had attained constant temperature, the samples were allowed to dissolve and the oxygen consumption was measured in the usual manner. Proper corrections were made for the slow progressive absorption of oxygen by nicotine in alkaline solution. The results, summarized in Table I, confirm the spectroscopic evidence that the material is cobaltomesoporphyrin.

No cobalt compounds with mesoporphyrin have been found described in the literature.

Other Preparations—Mesoporphyrin dimethyl ester was combined with cobalt and with manganese by essentially the methods already described. Both the cobalt and the manganese mesoporphyrin esters have spectra similar in type and in position of the bands to the spectra of the corresponding metalloporphyrins. Either compound is insoluble in aqueous alkaline solution unless nitrogenous base be added.

Chromium metalloporphyrins should be of interest, because the well known chromium ammines resemble closely the corresponding cobalt compounds, especially in exhibiting two valence states. Every attempt to prepare chromium mesoporphyrins, by any of the reported methods for metalloporphyrin synthesis, has

TABLE I

Oxygen Absorption by Cobaltomesoporphyrin

Samples dissolved in 5 ml. of 0.2 M NaOH + 0.5 ml. of nicotine at 30°.

Weight of sample (1)	Gas absorbed (0°, 760 mm. Hg) (2)	Gas absorbed (corrected)* (3)	Oxygen absorbed by sample (4)	Oxygen equivalent to sample (5)
gm.	*c.mm.*	*c.mm.*	*moles*	*moles*
0.00932	94.4	81.55	3.6×10^{-6}	3.7×10^{-6}
0.01222	118.3	105.45	4.7×10^{-6}	4.9×10^{-6}

* A solution of 5 ml. of 0.2 M NaOH + 0.5 ml. of nicotine without cobaltomesoporphyrin absorbed oxygen steadily for 2 hours. From this experiment was obtained the correction of 12.85 c.mm. (0°, 760 mm. of Hg) subtracted from Column 2 to yield Column 3.

failed. Hill (14) and Fischer, Treibs, and Zeile (13) have also reported failure. We have found no chromium porphyrins reported in the literature.

Copper and nickel mesoporphyrins, prepared as was the cobalt compound, gave no evidence of reversible oxidation and reduction.

Analyses

Each compound was ashed, preparatory to the analysis for cobalt and for manganese, respectively, by the same procedure. Samples weighed into quartz crucibles were warmed with 50 per cent sulfuric acid on a water bath for 2 hours. The crucibles were then placed in a cold muffle furnace, and were brought gradually

to 500°. After the evolution of fumes had ceased, the temperature was brought to 800° for a short time. The crucibles were cooled and the process was repeated, the furnace being kept at 500°, at which temperature there should be no conversion of sulfate to oxide. The residues proved completely soluble.

Manganese was determined by the bismuthate method, according to Cunningham and Coltman (15). The potassium permanganate solution was standardized just before use against sodium oxalate.

TABLE II

Analyses of Metalloporphyrins

Manganese	Weight of sample	Volume KMnO₄	Normality KMnO₄	Mn found	Mn calculated	Formula
	$gm.$	$ml.$		per $cent$	per $cent$	
Manganimeso-porphyrin	0.1482	9.81	0.1175	8.55	8.39	$C_{34}H_{35}O_4N_4MnCl$
	0.1508	10.03	0.1175	8.59	8.63	$C_{34}H_{35}O_4N_4MnOH$
	0.1511	10.14	0.1175	8.66	8.10	$C_{34}H_{35}O_4N_4MnC_2H_3O_2$

Cobalt	Weight of sample	Volume K₃Fe-(CN)₆	Normality K₃Fe-(CN)₆	Co found	Co calculated	Formula
	$gm.$	$ml.$		per $cent$	per $cent$	
Cobaltomeso-porphyrin	0.1361	4.24	0.04937	9.07		
	0.1443	4.47	0.04937	9.01	9.46	$C_{34}H_{36}O_4N_4Co$
	0.1435	4.44	0.04956	9.04		
	0.1425	4.40	0.04956	9.02		

Cobalt was determined by electrometric titration of cobaltous ion in the presence of ammonia, according to Tomíček and Freiberger (16). The ferricyanide solution was standardized against sodium thiosulfate and the latter was standardized against potassium biniodate.

Table II summarizes the results of the analyses. Since the manganese compound is certainly manganimesoporphyrin, the residual positive charge of the molecule in the solid state should be matched against some negative ion. The choice of OH⁻ produces good agreement between the found and calculated values, and the method of formation may be in its favor. The value found

for cobalt is low for cobaltomesoporphyrin, but the evidence for this formula has been given above. The presence of some free mesoporphyrin is not excluded, although none has been detected spectroscopically.

Electrometric Measurements

Technique—The equipment and technique in general follow those of Clark (17). To preserve the basis of comparison all electrode potentials have been referred to the normal hydrogen standard after the arbitrary conventions of Clark (18).

The tendency of alkaline metalloporphyrin solutions to foam during deaeration by bubbling nitrogen led to the use of a special form of titration vessel, stirred mechanically and sealed completely from air by a mercury seal. This permitted the nitrogen to be turned off during a titration, after a preliminary deaeration.

Reduction by dithionite ($Na_2S_2O_4$), under usual conditions, proved unsatisfactory. Von Zeynek (19) has shown that sulfurous acid attacks hemin in the presence of light, and some evidence of a similar reaction appeared in our studies. Accordingly, when dithionite was used the titration vessel was carefully shielded from light. The titrations were then quite satisfactory. In the more alkaline solutions the potential of the phthiocol system[1] is sufficiently low that reduced phthiocol can be used as a reducing agent (Ball (20)). It was used in most of the reductive titrations. Satisfactory titrations were also obtained with ferricyanide as oxidizing agent following reduction with hydrogen in the presence of colloidal palladium. Barron (3) has used Paal's palladium for a similar purpose, but we preferred the catalyst made with gum arabic, according to Skita and Franck (21), in order to avoid the introduction of protein material which might form a trace of protein metalloporphyrin.

Preliminary titrations in the presence of nicotine gave potentials drifting steadily negative. This difficulty was avoided by the use of two procedures. One was distillation of the nicotine *in vacuo* under nitrogen, which yielded a water-white product; the other was the exposure of the nicotine-metalloporphyrin mixture to the air for 1 or 2 days before titration. The presence

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[1] The dissertation includes a description of a modified method of synthesizing phthiocol.

of an easily oxidizable impurity is suggested. Distillation of pyridine over zinc and fractionation with a 60 cm. column failed to eliminate an impurity capable of being reduced. At the start of a titration it was necessary to wait until this material had been consumed and potentials became ·steady. Corrections for the amount of reducing agent so consumed are made when the titration data are analyzed by the method of Reed and Berkson (22), which was applied in every instance. The α-picoline, Eastman, was not further purified.

The pH values of the buffer-base solutions were determined by the use of the glass electrode, after the method outlined in the introductory paper (1). Since the characteristic potentials of the systems now under consideration were found independent of pH, the measurements are not as critical as for the cases studied by Davies (23) and by Vestling (24).

Potentials in Absence of Base—All attempts to titrate cobalt and manganese mesoporphyrins in the absence of coordinating base failed to give reliable data. The reductants are so slightly soluble in aqueous alkaline solution that precipitation began after a slight portion of reducing agent had been added to a solution in which the metalloporphyrin was 0.000025 M, and the electrode potential then continued to drift as if reductant were being continuously removed. This explanation seems adequate in the present instance but that another factor may be involved is suggested by the experience of Davies and Vestling with other metalloporphyrins.

Potentials in Presence of Base—When any suitable base is added to the cobalt or manganese metalloporphyrin system, the electrode potentials become remarkably steady, and tests can be made of some, if not all, of the relationships discussed in Paper I of this series (1). Results are reported with nicotine, pyridine, and α-picoline.

Titrations in the presence of piperidine yielded potentials drifting negative so rapidly that measurements were precluded.

Barron (3) and Davies (23) have shown that cyanide iron porphyrin systems may easily be titrated. Attempts to titrate cyanide cobalt- and manganese mesoporphyrins met with peculiar difficulties. No steady potentials were obtained except in the region poised by the phthiocol system, used as reducing agent.

No reduction appeared to be brought about by colloidal palladium and hydrogen.   Barron (3) also reports this for iron protoporphyrin and cyanide.   When solid cobaltomesoporphyrin was added to deaerated buffer solution containing cyanide ion, the metalloporphyrin did not dissolve.   Since there will appear some spectroscopic evidence that cyanide ion coordinates with cobaltimesoporphyrin, it may be suggested that it coordinates with this

TABLE III

α-Picoline Manganese Mesoporphyrin System

Titration of α-picoline manganimesoporphyrin with $Na_2S_2O_4$ in the dark at constant pH and constant α-picoline concentration.   Demonstration that $n = 1$.   α-Picoline = 0.93 M; total pigment = 2.5×10^{-4} M; $Na_2S_2O_4$ = ca. 1.36×10^{-3} N; pH of phosphate buffer + α-picoline = 7.60; $\mu = 0.22$; temperature = 30°; $d = 0.66$ ml.; 100 per cent reduction at $y = 7.99$ ml.

y	$y - d$	Reduction	$0.06011 \times$ $\log \frac{S_o}{S_r}$	E_h	E_b	Deviation from -0.2958
ml.	*ml.*	*per cent*		*volt*	*volt*	*volt*
1.50	0.84	11.46	+0.0534	−0.2452	(−0.2986)	−0.0028
2.00	1.34	18.28	+0.0391	−0.2573	−0.2964	−0.0006
2.50	1.84	25.10	+0.0285	−0.2674	−0.2959	−0.0001
3.00	2.34	31.92	+0.0198	−0.2760	−0.2958	0.0000
3.50	2.84	38.74	+0.0120	−0.2838	−0.2958	0.0000
4.00	3.34	45.57	+0.0046	−0.2913	−0.2959	−0.0001
4.50	3.84	52.38	−0.0025	−0.2985	−0.2960	−0.0002
5.00	4.34	59.21	−0.0097	−0.3055	−0.2958	0.0000
5.50	4.84	66.03	−0.0174	−0.3133	−0.2959	−0.0001
6.00	5.34	72.85	−0.0258	−0.3216	−0.2958	0.0000
6.50	5.84	79.67	−0.0357	−0.3308	−0.2951	+0.0007
7.00	6.34	86.49	−0.0485	−0.3427	(−0.2942)	+0.0016
7.60	6.94	94.68	−0.0752	−0.3641	(−0.2889)	+0.0069
Average					−0.2958	

form only.   If the reductant does not coordinate, its insolubility will make potentiometric titrations impossible.

Descriptions of Relations—Paper I (1) outlined various propositions, some of which will be submitted now to experimental tests.   For the sake of brevity we shall refer the reader to Paper I for definitions of the symbols and propositions now used.

Determination of p *and* n, *by Propositions I and II*—Tables

III and IV so well represent the results of numerous titrations as to make unnecessary the publication of the detail in all cases.

Rectification of the titration curves by the method of Reed and Berkson (22) according to Proposition XVI indicated clearly that $p = 1$ and $n = 1$, subject only to the slight and, we think, negligible doubt noted in Paper I.

TABLE IV   ·

Nicotine Cobalt Mesoporphyrin System

Titration of nicotine cobaltimesoporphyrin with reduced phthiocol at constant pH and constant nicotine concentration. Demonstration that $n = 1$. Nicotine = 0.30 M; total pigment = 2.5×10^{-4} M; phthiocol = ca. 6.4×10^{-4} M; pH of phosphate buffer + nicotine = ca. 11.4; $\mu = 0.45$; temperature = 30°; $d = 0.08$ ml.; 100 per cent reduction at $y = 7.92$ ml.

y	$y - d$	Reduction	$0.06011 \times \log \frac{S_o}{S_r}$	E_A	E_b	Deviation from -0.1992
ml.	*ml.*	*per cent*		*volt*	*volt*	*volt*
0.50	0.42	5.36	$+0.0750$	-0.1294	(-0.2044)	-0.0052
1.00	0.92	11.73	$+0.0527$	-0.1473	(-0.2000)	-0.0008
1.50	1.42	18.11	$+0.0394$	-0.1599	-0.1993	-0.0001
2.00	1.92	24.49	$+0.0294$	-0.1696	-0.1990	$+0.0002$
2.50	2.42	30.87	$+0.0211$	-0.1782	-0.1993	-0.0001
3.00	2.92	37.25	$+0.0136$	-0.1855	-0.1991	$+0.0001$
3.50	3.42	43.62	$+0.0067$	-0.1924	-0.1991	$+0.0001$
4.00	3.92	50.00	0.0000	-0.1992	-0.1992	0.0000
4.50	4.42	56.38	-0.0067	-0.2059	-0.1992	0.0000
5.00	4.92	62.76	-0.0136	-0.2128	-0.1992	0.0000
5.50	5.42	69.13	-0.0211	-0.2203	-0.1992	0.0000
6.00	5.92	75.51	-0.0294	-0.2286	-0.1992	0.0000
6.50	6.42	81.89	-0.0394	-0.2389	-0.1995	-0.0003
7.00	6.92	88.27	-0.0527	-0.2522	-0.1995	-0.0003
7.50	7.42	94.64	-0.0750	-0.2762	(-0.2012)	-0.0020
Average..					-0.1992	

The values of d were always small and varied with the base used. With pyridine and α-picoline d was always positive, but with nicotine sometimes negative, indicating reduction of some metalloporphyrin by the previously mentioned, reducing impurity.

The theory demands that the concentration of free base, [B], the pH value, and the ionic strength of the solution, as well as

the temperature, must remain constant if the constants p and n are to be evaluated. Estimates indicate that, when S_b remains constant, the change in [B] due to the difference in dissociation constants of the oxidized and reduced base metalloporphyrins is negligible. It will presently be shown that there should be no correction for pH changes during the titration of these systems, and only a slight correction, which may be neglected, for change in ionic strength during titration. The agreement among the values of E_b, when calculated by means of the coefficient 0.06011 for a 1 equivalent change, is, therefore, excellent evidence that p = 1 and n = 1 for all the systems reported here.

There remains the possibility that the metalloporphyrin systems in the absence of base have values of p and n greater than 1.

Effect of pH—The several individual titration curves, each analyzed by Proposition XVI, provide a series of values of E_b at different values of pH. The impracticability of making direct determinations of E'_0 make it unsafe to do more than reduce the values to a common value of S_b which has been taken to be 0.9 M, since this covers the cases. Corrections for variations in the ionic strength of the various buffer solutions have been made by means of an empirical equation that will be described later.

The assembled results are shown in Fig. 1 and clearly indicate that $\Delta E_h/\Delta$ pH = 0. While there appear slight discrepancies, we attribute them partly to experimental error and partly to the difficulty of reducing the data to a common basis. According to a corollary of Proposition X the relation demonstrated shows that within the range of pH studied there is no difference between oxidized and reduced base metalloporphyrins with respect to association with H^+ or OH^-. It does not show that both may not be associated with OH^-, for example.

Reduction of the data to a common basis of S_b does not provide a strictly logical basis of comparison for obvious reasons. None the less the relations made evident in Fig. 1 retain sufficient comparative value to be interesting.

The E'_0/pH curve of the phthiocol system (Curve B of Fig. 1), which is more negative than the metalloporphyrin systems in the alkaline range, crosses the curves for these systems at lower values of pH. For example, if a solution of manganimesoporphyrin and pyridine be reduced by phthiocol at pH 12, the acidifica-

tion of the solution to pH 7.5 should be more than sufficient to reverse the direction of the reaction. This has been confirmed experimentally for the cobalt and manganese metalloporphyrins in the presence of pyridine. This is another of the several examples exhibiting concretely the integration of acid-base and oxidation-reduction equilibria in a continuum.
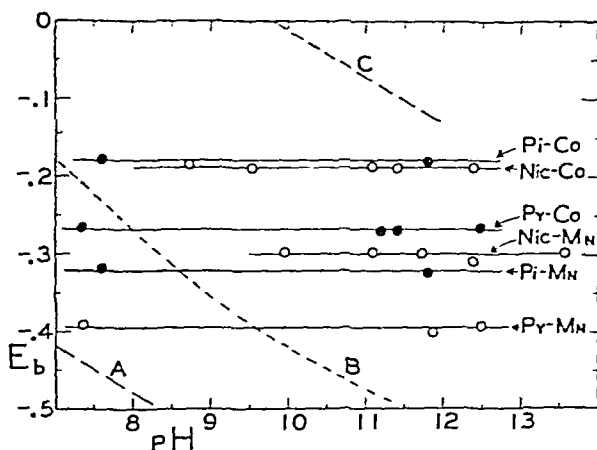
FIG. 1. Relation of potential E_b, at constant S_b, to pH. All data are reduced to $\mu = 0$, $S_b = 0.9$, $S = 2.5 \times 10^{-4}$ M, $S_o = S_r$, 30°. *Pi-Co*, α-picoline + cobalt mesoporphyrin; *Nic-Co*, nicotine + cobalt mesoporphyrin; *Py-Co*, pyridine + cobalt mesoporphyrin; *Nic-Mn*, nicotine + manganese mesoporphyrin; *Pi-Mn*, α-picoline + manganese mesoporphyrin; *Py-Mn*, pyridine + manganese mesoporphyrin. Curve A, hydrogen at 1 atmosphere; Curve B, phthiocol; Curve C, pyridine + iron protoporphyrin (heme).

Association Experiments—The extraordinary limitations of low solubility in the absence of base, already mentioned, and of incomplete association in the presence of enormous concentration of base, now to be described, prevented determination of the association constants by the method of establishing the limiting potentials and the complete association curves. The pyridine manganese mesoporphyrin system alone yielded data extensive enough to be of value in checking the laws of association. Even in this instance it was necessary to operate with the system only slightly reduced in order to obviate the difficulties arising from the precipi-

tation of manganomesoporphyrin at low pyridine concentrations. The less extensive data of other systems revealed nothing at variance with the conclusions which we shall draw, and their detailed consideration is omitted.

Fig. 2 summarizes the data. They are plotted in such a way as to facilitate the graphical analysis described in Paper I as Proposition V. The smooth curve of Fig. 2, constructed with the aid of the graphical analysis and the simplest assumptions, is theo-
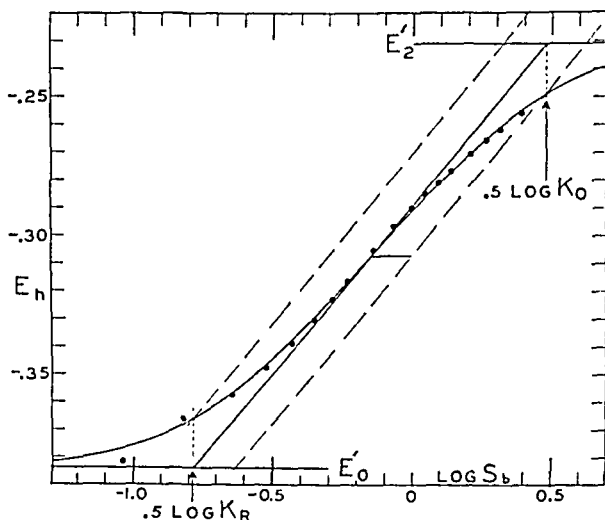
FIG. 2. Effect of adding pyridine to the manganese mesoporphyrin system, 5 per cent reduced. Initial total pigment, $[S]$, $= 2.5 \times 10^{-4}$ M; pH $= 12.35$; $\mu = 0.1$; temperature $= 30°$. The curve is drawn with $q = r = 2$, $K_O = 9.26$, $K_R = 2.64 \times 10^{-2}$, $E'_0 = -0.384$ volt, $E_2 = -0.231$ volt. E'_0 (at 50 per cent reduction and $\mu = 0$) $= -0.4830$ volt.

retical for the case in which $q = r = 2$ and the constants have the numerical values indicated in the legend.

Irrespective of the guidance that the theoretical curve may seem to impose, the following conclusions may be drawn.

The potential increases with increase of pyridine. This indicates that K_O is greater than K_R, according to Proposition III. The maximum of the slope, $dE_h/d (\log S_b)$, is very close to the value 0.1202. Could it be proved with certainty that the value of K_O/K_R were large enough to make Proposition VI strictly applicable, this would indicate that r has the value 2 ($0.0601r = 0.1202$). That K_O/K_R is sufficiently large is suggested by the fact that the

slope of the tangent does approach the indicated, integral multiple of 0.0601 and also by the spread between the points of maximal curvature.

Since the experimental curve departs from the maximal slope as S_b increases, q must be greater than 0 according to Proposition VIII.

Except for the possibility of an extraordinary distribution of the values of the dissociation constants, it remains fairly certain that $r = 2$. If K_R be in fact the product $K_{R_1}K_{R_2}$, the values of the individual constants are not very different.

The outstanding uncertainty is the conduct of the system at high concentration of pyridine and this concerns the coordination with manganimesoporphyrin. Obviously the data are not extensive enough for testing Proposition IV or IX. They are also insufficient for the adequate application of the rectification test of Proposition XV. None the less, neither the assumption (a) $\dot{q} = 1$, nor the assumption (b) $q = 2$, and no stepwise association, leads Proposition XV to a satisfactory result and this suggests stepwise association. Of this all that can be said is the following. Once the general form of the association curve has been satisfactorily defined with respect to first order approximation as it has been by the curve of Fig. 2, it becomes possible to reproduce this curve rather closely by preserving the same product $K_o = K_{o_1}K_{o_2}$ with various distributions of the individual values of K_{o_1} and K_{o_2}.

This shows clearly the limitations of the potentiometric method when the properties of a system restrict the extent of exploration. Particular attention may be called to this, since other limitations are inherent in the interpretation of spectrophotometric data and conclusions will have to be pieced together from the results of both methods.

Effect of Ionic Strength of Solution on Potential—Pending the development of the chief features of the metalloporphyrin systems it was thought advisable to give but secondary attention to the effect of variations in the ionic strength. Such variations do affect the oxidation-reduction potential. Significant results may attend a more exhaustive investigation than the following orienting experiment.

With the equations given in Paper I it may be shown that

when p $= m = n = 1$, $r = q = 2$, and $S_o/S_r =$ constant, the potential should vary with activity coefficients as follows: When [B] $= 0$

$$E_h \propto \frac{RT}{F} \ln \frac{\gamma_O}{\gamma_R} \qquad (1)$$

When [B] $\gg K_O$ and K_R

$$E_h \propto \frac{RT}{F} \ln \frac{\gamma_{OB_2}}{\gamma_{RB_2}} \qquad (2)$$

Between these extremes E_h is a complicated function of the four activity coefficients mentioned and of γ_B^2.   If z_r is the valence of the reductant, and z_o the valence of the oxidant, the limiting law of Debye and Hückel would give, at 30°,

$$E_h \propto 0.03006\,(z_r^2 - z_o^2)\,\sqrt{\mu} \qquad (3)$$

for either Equation 1 or 2.

The absence of a pH effect in the systems under discussion suggests, but does not prove, that there is no change of valence when a neutral substance (like pyridine or nicotine in alkaline solution) coordinates with the metalloporphyrins.   Therefore we may proceed tentatively with Equation 3.

There are two carboxyl groups in mesoporphyrin.   Let it be assumed that these two groups as they occur in the metalloporphyrin are completely ionized and function only as ionized groups at high pH values.   If either of the ions Mn^{++} or Mn^{+++} replaces 2 protons at the coordination center, the net charge of manganomesoporphyrin should be two (negative kind) and of manganimesoporphyrin should be one (negative kind).   Let the same charges be assumed for the nicotine complexes.   Accordingly, and if all conditions be constant except the ionic strength of the solution, the slope of the limiting Debye-Hückel relation should be

$$\frac{dE_h}{d\sqrt{\mu}} = 0.0902 \qquad (4)$$

To a solution of manganese mesoporphyrin about 23 per cent reduced with phthiocol, 0.005 M with respect to sodium hydroxide, and 0.3 M with respect to nicotine, there were added successive

portions of a solution of potassium chloride, containing the same concentration of nicotine as did the original solution after the addition of the phthiocol. Fig. 3 summarizes the experimental data.

The dotted line of Fig. 3 is drawn with slope $\Delta E_h/\Delta\sqrt{\mu} = 0.0902$ corresponding to $z_r = 2$ and $z_o = 1$. The curve through the experimental points was drawn with the extended equation
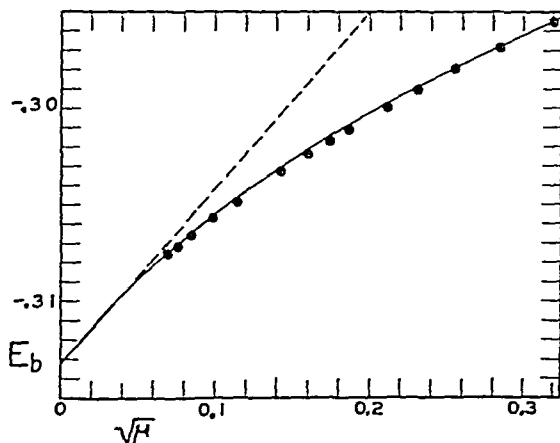
FIG. 3. Relation of potential to ionic strength in the nicotine manganese mesoporphyrin system. The system was about 23 per cent reduced with phthiocol. Total pigment = 2.76×10^{-4} M; nicotine = 0.30 M; temperature = 30°; straight line $\Delta E/\Delta\sqrt{\mu} = 0.09$.

of Debye and Hückel and the arbitrary value 6×10^{-8} cm. for a, the "ionic diameter." The complete equation is

$$E_h \propto \frac{0.0902\sqrt{\mu}}{1 + 1.98\sqrt{\mu}} \qquad (5)$$

To use the apparent approach to the predicted limiting slope as an argument in favor of the specified ionic charges it will have to be shown that the natures of the molecules under consideration do not seriously invalidate the applicability of the limiting law of Debye and Hückel.

Log Transmittance Curves—The curves of various cobalt mesoporphyrin systems are presented in Figs. 4, 5, 7, and 8.

Since spectrophotometric characterization of porphyrin derivatives is valuable in furnishing constants of reference, Table V

records the molar extinction coefficients, ϵ, at specific wavelengths, for the cobalti- and manganimesoporphyrins. These constants are calculated according to the equation

$$-\log T = \epsilon cl$$

where T is transmittance, c is molar concentration, and l is cell length in cm.

It is worth remarking, with respect to these curves, that of the bases tested only cyanide produces a striking change in the location of the absorption bands. This is in contrast to the behavior of the iron porphyrin systems. Pauling and Coryell

TABLE V

Molar Extinction Coefficients

Concentration of pigment $\times 10^5$	λ	$-\log T$	ϵ
Manganimesoporphyrin. NaOH = 0.01 M; cell length = 4 cm.; slit width = 0.1 mm.			
	$m\mu$		
4.95	552	1.2676	6.4×10^3
0.99	552	0.3372	8.5×10^3
Cobaltimesoporphyrin. NaOH = 0.005 M; cell length = 4 cm.; slit width = 0.1 mm.			
2.535	545	0.7894	7.8×10^3
2.415	545	0.7293	7.6×10^3
2.488	545	0.7359	7.4×10^3
2.488	560	1.2017	12.1×10^3
2.488	525	1.1654	11.7×10^3

(25) have pointed out that there is a correlation between the type of spectrum and the type of metal-porphyrin bond, as revealed by magnetic measurements, in ferri- and ferroprotoporphyrin, in a number of their base complexes, and in nickel protoporphyrin. This would suggest that in cobaltimesoporphyrin, and perhaps in other systems observed, there is no change in metal-porphyrin bond type upon the addition of nicotine, pyridine, or α-picoline.

Viewing the log transmittance curves upside down in order that we may speak of absorption "peaks" one notices that cobaltomesoporphyrin, nicotine cobaltomesoporphyrin, and cyanide

cobaltomesoporphyrin each has the sign of a "foot-hill" beside the "peak" and at lower wave-length. On the other hand, in the case of the manganomesoporphyrin (Fig. 10; see "Supplementary note") a similar "foot-hill" is at higher wave-length.

The curves for cobaltimesoporphyrin in the presence and absence of nicotine, shown in Fig. 4, coincide so nearly that they cannot be used in the study of the association equilibrium ac-
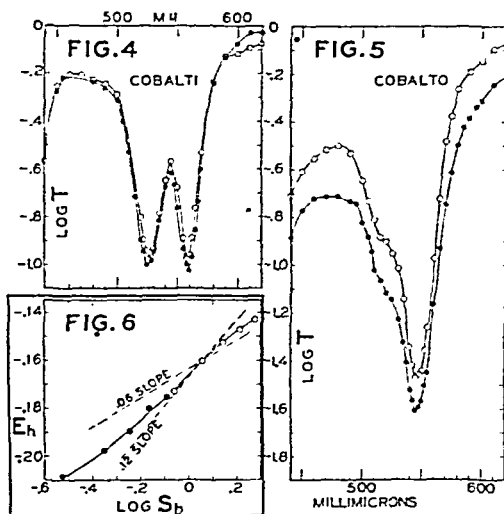
FIG. 4. Log transmittance curves. O represents nicotine cobaltimesoporphyrin IX. $S = 8.96 \times 10^{-6}$ M; $S_b = 0.25$ M; NaOH $= 0.02$ M; 10 cm. tube. ● same without nicotine.

FIG. 5. Log transmittance curves. O represents nicotine cobaltomesoporphyrin IX. $S = 8.96 \times 10^{-6}$ M; $S_b = 0.25$ M; NaOH $= 0.02$ M; 10 cm. tube. ● same without nicotine.

FIG. 6. Relation of E_h to log S_b at constant degree of reduction. ● represents cobalt mesoporphyrin + nicotine; O cobalt mesoporphyrin + pyridine.

cording to the method outlined in Paper I (1). The same is true for the pyridine and α-picoline systems, whose curves are essentially identical with the curves in Fig. 4. The curve for cobaltomesoporphyrin (Fig. 5) may not be strictly comparable with that for cobaltomesoporphyrin plus nicotine, since the concentration is near the solubility limit of the free cobaltomesoporphyrin, and a solid phase may have separated in particles large

enough to alter the transmittance. Assuming that the observed differences are not artifacts, they remain too small to be used in the study of the association with the instrument available.

The addition of cyanide to cobaltimesoporphyrin does produce a marked alteration in the curve, as shown in Fig. 7. The differences should be capable of use in studying the course of association to form a cyanide cobaltimesoporphyrin, yet we have not succeeded in analyzing the data according to any equilibrium equa-
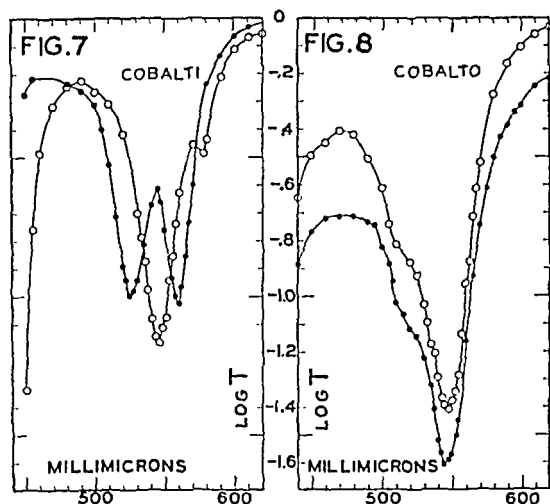
Fig. 7. Log transmittance curves. O represents cyanide cobaltimesoporphyrin. $S = 8.96 \times 10^{-6}$ M; $S_b = 0.008$ M; NaOH $= 0.02$ M; 10 cm. tube. ◉ same without cyanide.

Fig. 8. Log transmittance curves. O represents cyanide cobaltomesoporphyrin. $S = 8.96 \times 10^{-6}$ M; $S_b = 0.008$ M; NaOH $= 0.02$ M; 10 cm. tube. ◉ same without cyanide.

tion. Fig. 8 shows that the curves for the two reduced systems are similar.

Comparison of Figs. 4 to 6 is of special interest. The formation of a nicotine cobaltomesoporphyrin is indicated by the change of potential (*cf.* Fig. 6). Fig. 6 also shows evidence of a combination between cobaltomesoporphyrin and pyridine. On the other hand Fig. 4 suggests no combination of the cobalti compound with nicotine and less quantitative studies suggest little distinct spectral evidence of the combination with pyridine. Now potentiometric data could render a decision on this elementary question were

the properties of the system such as to permit extensive potentiometric measurements and this they do not permit. We hope that we are not overlooking something of significance when, for the sake of simplicity and on the basis of instability of potentials and formation of precipitates, we reject from Fig. 6 abnormally high potentials observed at the lower concentrations of pyridine. The residual and more reliable data which alone are shown suggest a definite departure from the "0.12 slope" and this can be taken as presumptive evidence that $q > 0$ (see Proposition VIII, but note the underlying assumptions).

SUMMARY

Manganese mesoporphyrin and cobalt mesoporphyrin have been prepared and characterized. The latter is believed to be a new compound.

Potentiometric titration curves of systems containing each one of these compounds and pyridine, or nicotine, or α-picoline, have been analyzed by an objective method and have yielded the conclusions that there is no evidence of polymerization and that 1 equivalent is concerned in the oxidation-reduction process. Numerical values of the characterizing potentials are recorded.

All other conditions being the same within narrow experimental limitations, dE_h/d pH $= 0$ in all the systems.

Limitations imposed by the properties of one or another of the components have prevented the determination of the full course of the association between these metalloporphyrins and any one of the bases mentioned; but sufficient of the course of association between pyridine and mixed mangani- and manganomesoporphyrin has been followed to draw the following conclusions. It is certain that pyridine associates better with manganomesoporphyrin than with manganimesoporphyrin. It is reasonably certain that 2 molecules of pyridine associate with 1 of manganomesoporphyrin and that at least 1 pyridine associates with manganimesoporphyrin. It is not improbable that 2 pyridines associate with manganimesoporphyrin. The best agreements so far found between the data and a theoretical formulation are summarized by the following numerical values of constants defined in Paper I: $p = m = n = 1$, $q = r = 2$, $K_o = 9.26$, $K_R = 0.0264$, $E'_0 = -0.4830$ volt, $E_2 = -0.3300$ volt. The way is opened

for a formulation in terms of stepwise association between pyridine and manganimesoporphyrin.

The simplified equation of Debye and Hückel, relating activity coefficients to ionic strength, has been extended to describe the effect of changing ionic strength on the potential of the nicotine manganese mesoporphyrin system. While the results of the experimental test are not decisive, they suggest that, if factors not now considered can be shown not to invalidate the treatment, the net charge of nicotine manganimesoporphyrin is one and that of the mangano compound is two.

Spectral data for several of the systems are recorded.

SUPPLEMENTARY NOTE

By W. MANSFIELD CLARK

Taylor's potentiometric data could not decide the conduct of manganimesoporphyrin with pyridine and the spectrophotometric equipment with which he attempted to solve this and similar questions did not meet the requirements of precision that the problem demanded. Therefore he generously consented to withhold publication of his dissertation pending my attempt to meet the situation by constructing a photoelectric spectrophotometer.

The instrument used is described in Paper V.

Figs. 9 and 10 show log transmittance curves of the systems concerned. Fig. 11 records one of several sets of data for the association of manganimesoporphyrin and pyridine and one of several sets of data for the association with manganomesoporphyrin. None of these curves yielded satisfactory results by the estimates of Proposition XII or by the rectification method of Proposition XIV and therefore interpretations of the curves require the exercise of judgment.

Even if the mangano system follows a simple law, it will be noted that the maximal difference, $\epsilon'_2 - \epsilon'_1$, is so small as to require an extreme precision of measurement to satisfy any requirement exactly. Indeed, it is surprising that the data align as well as they do. What is of first order importance is that the mid-point, estimated to occur at $\log S_b = -0.68$, is not seriously inconsistent with Taylor's first order approximation of K_R. His value, estimated on the basis of $r = 2$, would predict that the half transformation point should occur where shown by the center of the

dotted curve of Fig. 11. Corrections for the approximation $S_b \doteq [B]$ are not significant. Were it true that K_R should be the product of K_{R_1} and K_{R_2} and that the values of the latter differ slightly, there is opened the possibility of explaining qualitatively the departure of the spectral curve from the theoretical curve for $r = 2$. Such a gratuitous, *ad hoc* assumption is mentioned only because it and the limitations of a theoretical treatment to
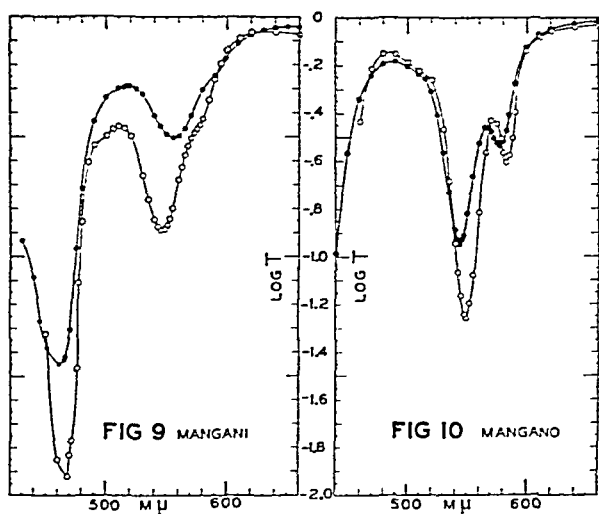
FIG. 9. Log transmittance curves for manganese mesoporphyrin IX. Approximately 0.053 gm. + 0.025 M KOH per liter in a 1 cm. tube was used. ● represents manganimesoporphyrin; ○ pyridine manganimesoporphyrin (1.24 M pyridine).

FIG. 10. Log transmittance curves for manganese mesoporphyrin IX. Approximately 0.053 gm. + 0.025 M KOH per liter in a 1 cm. tube was used. ● represents manganomesoporphyrin; ○ pyridine manganomesoporphyrin (1.24 M pyridine).

two absorbing species do place the spectral data under suspicion except with regard to the order of magnitude of K_R. In this they confirm Taylor's potentiometric estimate as well as could be expected from the application of Proposition XIII.

Use of Propositions XII and XIV in analyzing the spectral data of the mangani system (Fig. 11) yielded unsatisfactory results the best of which signifies that $q = 3$ on the basis of no

stepwise association.   The curve drawn on this basis is the dotted line of Fig. 11.   Unless the whole picture, as it has been pieced together up to this point, be reconstructed, this value of q is impossible, since it and the attendant estimates of K_R and K_O would lead the potentiometric curve over a "hill" to declining values of E_h with increase of S_b.   Such was not found and the assumption must be rejected.   Furthermore, interpretations of the spectral data are put under suspicion by the observation that Beer's law
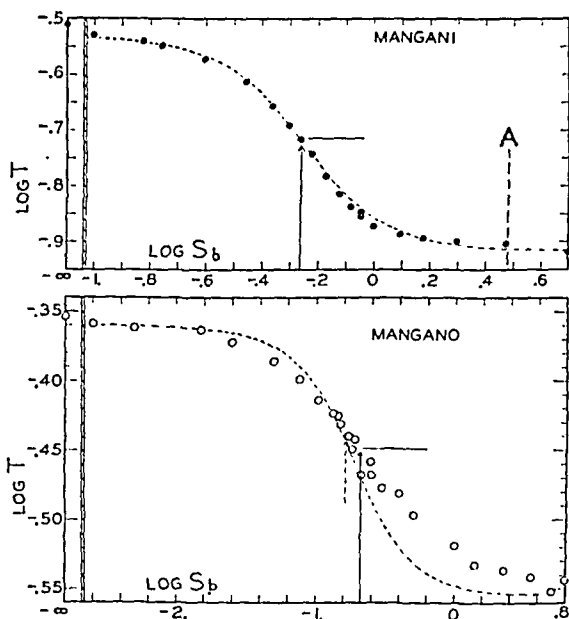
FIG. 11. Relation of log transmittance to log [pyridine]. S_b = molar concentration of pyridine.   Manganimesoporphyrin, approximately 0.053 gm. + 0.05 M KOH per liter in a 1 cm. tube, mμ 548; manganomesoporphyrin IX, approximately 0.022 gm. + 0.01 M KOH per liter in a 1 cm. tube, mμ 550.

was not followed strictly by solutions of manganimesoporphyrin (see Table VI).   This may be an evidence that there is change in state of aggregation of the manganimesoporphyrin.   Evidence suggesting this has escaped notice previously for reasons already noted.

    Examination of the consequences of the apparent failure of Beer's law in the hope of correcting the raw data preparatory to rectification by Proposition XIV was judged to be a case of "strain-

ing at a gnat and swallowing a camel" when the implications of the following remarkable fact were seen.

The order of magnitude of $\log S_b$ at which the spectral data indicate half transformation of manganimesoporphyrin to a pyridine complex is entirely inconsistent with Taylor's first, tentative estimate of the value of K_0 made on the basis that $q = 2$. That estimate would demand the curve of Fig. 11 to be displaced far to the right with the center at $\log S_b = +0.48$ (Point A). Conversely, use of the spectrophotometrically estimated constant would throw

TABLE VI

Effect of Dilution on Transmittance of Manganimesoporphyrin

Solution A, approximately 5×10^{-4} M manganimesoporphyrin in 0.02 M KOH; diluted with 0.02 M KOH; cell length=1 cm. Assume $-\log T = \epsilon C$.

Weight, Solution A per 100 mL.	$-\text{Log } T$	$\left(\dfrac{-\text{Log } T}{\text{Weight}}\right)$	Approximate concentration $\times 10^4$	Log concentration	ϵ
gm.					
0.4231	0.0144	0.0340	2.115	−5.675	6809
1.229	0.0411	0.0334	6.145	−5.211	6688
2.441	0.0806	0.0330	12.205	−4.914	6604
4.885	0.1553	0.0318	24.43	−4.613	6357
9.765	0.2983*	0.0305	48.83	−4.311	6109
14.599	0.4396*	0.0301	73.00	−4.137	6022

* Tyndall beam distinct.

a theoretical association curve quite off the track followed by Taylor's potentiometric data.

Taylor has opened the question of whether or not there is a stepwise association and has noted that if such be the case the theoretical curve of his Fig. 2 can be very nearly reproduced by preserving his value of K_0 but redefining K_0 as the product $K_{0_1}K_{0_2}$, to the separate constants of which are assigned empirically various values.

Now the analysis of spectral data according to the methods outlined in Paper I (1) depend upon the assumption that there are only two absorbing species. Stepwise association calls for more than two absorbing species.

But let us make the following assumptions *ad hoc*: (a) that absorption by the species OB_2 is only slightly greater than ab-

sorption by the species OB; (b) that K_{o_1} is considerably greater than K_{o_2}.

The result would be a distortion of the spectral curve but the center of this curve would be approximately where log K_{o_2} = log S_b. On these assumptions K_{o_2} is estimated to be 0.54. Let this be applied to the potentiometric curve. Taylor's estimate of K_o is 9.26. If this be $K_{o_1}K_{o_2}$ and K_{o_2} be 0.54, K_{o_1} = 17.15. With these values and Taylor's K_R = 0.0264 his curve (Fig. 2) is so nearly reproduced that only slight empirical readjustments of the constants would be required to make the new theoretical curve fit the data as well as the old. The specifications for this first trial are

$$E_h = E'_0 + 0.0601 \log \frac{K_{o_1}K_{o_2}}{K_R} + 0.0601 \log \frac{K_R + S_b^2}{K_{o_1}K_{o_2} + K_{o_2}S_b + S_b^2}$$

where E'_0 = -0.3869 (uncorrected to standard conditions), K_R = 0.0264 (Taylor), K_{o_1} = 17.15, K_{o_2} = 0.54 (spectral estimate), $K_{o_1}K_{o_2}$ = 9.26 (Taylor).

Furthermore, the adoption of the spectral value but with the assumption that q = 1 will not give a potentiometric curve fitting Taylor's data.

Although this hypothesis roughly establishes consistency between the potentiometric and spectrophotometric data, it is not certain that it is the only way to do so. On the other hand the case illustrates admirably how misleading might be spectrophotometric data when considered alone.

Obviously the properties of these systems are poorly adapted to the experimental elucidation of some features of association between metalloporphyrins and bases and therefore we shall do well to turn to other systems for developments.

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METALLOPORPHYRINS

III. COORDINATION OF NITROGENOUS BASES WITH IRON MESO-, PROTO-, AND HEMATOPORPHYRINS*

By T. HARRISON DAVIES

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The potentiometric study of heme in coordination with nitrogenous bases, initiated by Conant and his students, has been greatly extended by Barron (1). Barron and Hastings (2) announced a summary of their earlier data when a plan of a more general study of metalloporphyrins was being drawn in this laboratory. They graciously agreed that we should continue with the subject and give special attention to the effects of changing independently the metal and porphyrin components. It was our original intention to repeat some of their work only to profit by their invaluable experience but as there developed from the study of other systems the evidences of unsolved problems, we have felt justified in recovering some of the ground examined by Barron. Data for systems containing iron protoporphyrin IX, such as were obtained by Barron, are now compared with analogous data for systems containing iron mesoporphyrin IX and the corresponding iron hematoporphyrin, but within the restricted limits set by the properties of those systems that are adapted to potentiometric and spectrophotometric study.

Preparations

Preparations of hemin chloride and of mesoporphyrin IX have been described in Paper II of this series (see Taylor (3)).

The first of these materials was, as a matter of course, the form in which we employed iron protoporphyrin IX.

Ferrimesoporphyrin chloride was prepared by the method of Fischer and Stangler (4) from mesoporphyrin hydrochloride.

Hematoporphyrin dimethyl ether was prepared according to the method of Küster *et al.* (5) except in minor detail. It was recrystallized from 90 per cent methyl alcohol, and used in the preparation of iron hematoporphyrin chloride.

Ferrihematoporphyrin chloride proved difficult to prepare in pure state. Recrystallized hematoporphyrin dimethyl ether (1.2 gm.) was dissolved in 70 per cent acetic acid (75 ml.) saturated with sodium chloride. To this solution, held at 90°, was added an acetic acid solution of ferrous acetate until the color changed from red to brown: After further heating at 90° for 10 minutes the solution was cooled. The precipitate was collected and washed with a little methyl alcohol containing hydrochloric acid. The yields of several preparations averaged 0.7 gm. The method did not yield uniform preparations. Several black samples were semicrystalline but proved to be mixtures. Only one preparation, a brown amorphous material, when treated with cyanide gave a titration curve typical of a compound pure with respect to titratable material. In preparation of the metalloporphyrin from the dimethyl ether, the latter presumably should be hydrolyzed before it coordinates with the metal ion. It is conceivable that a portion of the hematoporphyrin dimethyl ether may coordinate, oxidize, precipitate, and thus escape hydrolysis. It might appear better to prepare hematoporphyrin itself and to form the metalloporphyrin directly from this; but in the first place it is difficult to prepare in pure state and in the second place its use in hot acetic acid solutions sufficiently strong to favor the formation of the desired crystals might lead to protoporphyrin (Fischer and Zeile (6)). The latter would affect our potentiometric measurements more seriously than would miscellaneous impurities of other kinds.

The identity of our preparations of ferrihematoporphyrin with those described by Fischer, Küster, and others is reasonably well established by the similarity of the methods of preparation, by the close correspondence between the spectral absorptions, and, in so far as analysis is valuable in such cases, by the agreement between found and calculated iron content.

The three metalloporphyrin preparations were analyzed for iron by the method of Klumpp (7). His procedure was altered in a few details for convenience. The titrations were performed under nitrogen instead of the recommended carbon dioxide. The titanous sulfate solution was standardized against a permanganate solution instead of against a ferric ammonium sulfate solution. The results appear in Table I.

TABLE I

Analyses of Metalloporphyrins for Iron

	Weight of sample	Ti₂(SO₄)₃ solution required	Normality of Ti₂(SO₄)₃ solution	Fe found	Fe calculated	Formula
	$mg.$	$ml.$		per $cent$	per $cent$	
Ferriprotoporphyrin chloride	89.6	5.82	2.232×10^{-2}	8.10	8.57	$C_{34}H_{32}O_4N_4FeCl$
	92.9	6.10	2.232×10^{-2}	8.18		
Ferrimesoporphyrin chloride	98.1	6.45	2.232×10^{-2}	8.19	8.52	$C_{34}H_{36}O_4N_4FeCl$
	96.4	6.32	2.232×10^{-2}	8.17		
Ferrihematoporphyrin chloride	43.2	7.88	8.03×10^{-3}	8.14	8.12	$C_{34}H_{36}O_6N_4FeCl$

Description of Results

Series of data have been analyzed in terms of the several propositions set forth in Paper I (8) and for brevity these propositions are referred to here by number.

Potentiometric Measurements

Titrations in Absence of Coordinating Base—

$$O_n + ne \rightleftharpoons pR_m$$
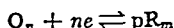
The electrode potentials of mixtures of ferrimesoporphyrin and ferromesoporphyrin exhibit drifts as if reductant were being withdrawn continuously from the field. Since precipitates of the bright red reductants were observable and the entire picture conformed to what Taylor found in his study of the cobalt and manganese mesoporphyrins, it seemed reasonable to attribute

instability of potential to progressive precipitation of the re-
ductant. On the other hand the same electrode behavior was
observed with alcohol solutions in which there appeared adequate
solubility, and Vestling, working with the water-soluble ferro-
coproporphyrin, encountered similar positive drifts. Barron (1)
reports success in electrode measurements with iron protopor-
phyrin but emphasizes the lack of strict reversibility of this
system. Since we have not yet succeeded in obtaining a wholly

TABLE II

Cyanide Iron Hematoporphyrin System

Titration of cyanide ferrihematoporphyrin with reduced phthiocol at
constant pH and constant cyanide concentration. Demonstration that
$n = 1$. NaCN $= 1.63 \times 10^{-2}$ M; total pigment $= 1.72 \times 10^{-4}$ M; phthiocol $\doteqdot$
1.5×10^{-3} N; pH of phosphate buffer $= 10.77$; $\mu = 0.18$; $d = +0.22$ ml.;
temperature $= 30°$; 100 per cent reduction at $y = 5.3$ ml.

y	$y - d$	Reduction	$0.06011 \times \log \frac{S_r}{S_o}$	E_h	E_b	Deviation from average
ml.	*ml.*	*per cent*		*volt*	*volt*	*volt*
1.0	0.78	15.354	-0.0446	-0.1536	-0.1982	0.0021
1.3	1.08	21.259	-0.0342	-0.1622	-0.1964	0.0003
1.58	1.36	26.771	-0.0263	-0.1699	-0.1962	0.0001
1.88	1.66	32.677	-0.0189	-0.1775	-0.1964	0.0003
2.18	1.96	38.582	-0.0121	-0.1841	-0.1963	·0.0002
2.48	2.26	44.488	-0.0054	-0.1906	-0.1960	-0.0001
2.78	2.56	50.393	0.0004	-0.1966	-0.1963	0.0002
3.08	2.86	56.299	0.0067	-0.2030	-0.1963	0.0002
3.38	3.16	62.204	0.0130	-0.2092	-0.1961	0.0000
3.68	3.46	68.110	0.0198	-0.2158	-0.1959	-0.0002
3.98	3.76	74.015	0.0273	-0.2228	-0.1965	-0.0004
4.58	4.36	85.826	0.0470	-0.2490	-0.1920	-0.0041
Average					-0.1961	

reliable set of potentiometric measurements with any one of the
metalloporphyrin systems in the *absence* of coordinating bases,
we shall not report the measurements made in this very important
part of the field. In some instances a fair estimate of the charac-
teristic potentials may be calculated with certain assumptions
and, should the accumulating data converge, it may be possible
then to see more clearly the reasons for the electrode instability,
and to subject these to special experimental tests.

Titrations in Presence of Coordinating Bases—

$$O_nB_q + ne \rightleftharpoons pR_nB_r$$
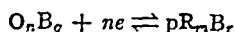
When certain bases are added to buffered solutions containing ferro- and ferriporphyrins, the electrode potentials become

TABLE III

Pyridine Iron Mesoporphyrin System

Titration of pyridine ferrimesoporphyrin with reduced phthiocol in alcohol-buffer mixture at constant pH and constant pyridine concentration. Demonstration that $n = 1$. Solvent, 47.5 per cent ethanol; pyridine = 4.56×10^{-1} M; total pigment = 1.47×10^{-4} M; phthiocol $\doteqdot 1.6 \times 10^{-3}$ N; pH of phosphate buffer + ethanol + pyridine = 11.22; $\mu = 0.19$; temperature = 30°; $d = 0$; 100 per cent reduction at $y = 4.64$ ml.

y	Reduction	$0.06011 \times \log \frac{S_r}{S_o}$	E_h	E_b	Deviation from average
ml.	per cent		volt	volt	volt
0.2	4.314	−0.0809	−0.0761	−0.1570	0.0006
0.4	8.629	−0.0616	−0.0959	−0.1565	0.0010
0.6	12.944	−0.0498	−0.1075	−0.1573	0.0003
0.9	19.417	−0.0372	−0.1201	−0.1573	0.0003
1.2	25.889	−0.0275	−0.1303	−0.1578	−0.0002
1.5	32.362	−0.0193	−0.1384	−0.1577	−0.0001
1.8	38.834	−0.0119	−0.1458	−0.1578	−0.0002
2.1	45.307	−0.0049	−0.1527	−0.1576	0.0000
2.4	51.779	0.0018	−0.1597	−0.1579	−0.0003
2.7	58.252	0.0087	−0.1668	−0.1581	−0.0005
3.0	64.724	0.0158	−0.1739	−0.1580	−0.0007
3.3	71.197	0.0236	−0.1814	−0.1578	−0.0002
3.6	77.669	0.0325	−0.1902	−0.1577	−0.0001
3.9	84.142	0.0435	−0.1996	(−0.1561)	0.0015
4.2	90.614	0.0592	−0.2111	(−0.1520)	0.0056
4.5	97.08	0.0911	−0.2295	(−0.1384)	0.0192
Average..................................				−0.1576	

emarkably steady, provided attention is given to conditions that maintain both the oxidized and reduced metalloporphyrins in solution. The very low solubility in aqueous solution of ferromesoporphyrin and its base complexes was particularly troublesome and because of it aqueous alcoholic solutions were employed. In the use of nicotine, rapid negative potential drifts

were encountered similar to those noted by Taylor with his metalloporphyrins and nicotine. These were eliminated by the procedure employed by him.

Tables II to VI exhibit fairly representative titration data and make it unnecessary to tabulate the data of all cases. A summary will be given. Analysis of the results by Propositions

TABLE IV

Nicotine Iron Protoporphyrin System

Titration of nicotine ferriprotoporphyrin with reduced phthiocol in alcohol-buffer mixture at constant pH and constant nicotine concentration. Demonstration that $n = 1$. Solvent, 47.5 per cent ethanol; nicotine = 3.53×10^{-1} M; total pigment = 1.44×10^{-4} M; phthiocol $\doteqdot 1.59 \times 10^{-3}$ N; pH of borate buffer = 10.232; $\mu = 0.05$; temperature = 30°. 100 per cent reduction at $y = 4.682$ ml.

y	$y - d$	Reduction	$0.06011 \times \log \frac{S_r}{S_o}$	E_h	E_b	Deviation from average
ml.	*ml.*	*per cent*		*volt*	*volt*	*volt*
0.2	0.32	6.663	−0.0689	0.0605	(−0.0084)	−0.0010
0.4	0.52	10.828	−0.0550	0.0476	−0.0074	−0.0000
0.7	0.82	17.076	−0.0413	0.0340	−0.0073	0.0001
1.0	1.12	23.323	−0.0311	0.0239	−0.0072	0.0002
1.3	1.42	29.571	−0.0227	0.0154	−0.0073	0.0001
1.7	1.82	37.900	−0.0129	0.0053	−0.0076	−0.0002
2.1	2.22	46.230	−0.0040	0.0030	−0.0070	0.0004
2.5	2.62	54.560	0.0048	−0.0124	−0.0076	−0.0002
2.9	3.02	62.890	0.0138	−0.0214	−0.0076	−0.0002
3.3	3.42	71.220	0.0237	−0.0314	−0.0077	−0.0003
3.7	3.82	79.550	0.0355	−0.0433	−0.0078	−0.0004
4.1	4.22	87.880	0.0517	−0.0589	−0.0072	0.0002
4.4	4.52	94.127	0.0725	−0.0800	−0.0075	−0.0003
4.6	4.72	98.292	0.1058	−0.1057	(−0.0001)	0.0073
Average...					−0.0074	

I, II, and XVI led to objective evaluations of p and n, and also of d, the quantity of reducing agent oxidized by impurities. The correction for the latter is always small and even in the case of the iron hematoporphyrin sample, under suspicion because of preparative difficulties, it demonstrates that the bulk of the material titrated as would a substance supplying a homogeneous

oxidation-reduction system. The correction factors of numerous titrations of the same metalloporphyrin preparations with different bases and with different preparations of the same base varied. Accordingly, we interpret the d value to be a measure of the oxidizing impurity introduced to the solution with the relatively excessive quantities of base.

Comparison of Tables IV and V reveals a remarkable contrast between nicotine iron protoporphyrin in *aqueous* solution and the other cases. While titration data of other systems and even of

TABLE V

Nicotine Iron Protoporphyrin System

Titration of nicotine ferriprotoporphyrin with reduced phthiocol at constant pH and constant nicotine concentration. Demonstration that $n = 2$. Nicotine = 2.9×10^{-1} M; total pigment = 4.76×10^{-4} M; pH of phosphate buffer + nicotine = 10.65; $\mu = 0.16$; $d = 0.80$ ml.; 100 per cent reduction at $y = 8$ ml.; temperature = $30°$.

v	$v - d$	Reduction	$0.03006 \times \log \frac{S_r}{S_o}$	E_h	E_b	Deviation from average
ml.	ml.	per cent		volt	volt	volt
1.0	0.2	2.777	−0.0461	−0.0089	(−0.0550)	−0.0065
1.5	0.7	9.722	−0.0295	−0.0201	(−0.0496)	−0.0011
2.0	1.2	16.666	−0.0209	−0.0273	−0.0483	0.0003
2.5	1.7	23.611	−0.0153	−0.0330	−0.0484	0.0002
3.0	2.2	30.550	−0.0107	−0.0378	−0.0485	0.0000
4.0	3.2	44.444	−0.0029	−0.0455	−0.0485	0.0001
5.0	4.2	58.330	0.0044	−0.0529	−0.0485	0.0000
6.0	5.2	72.222	0.0125	−0.0613	−0.0488	−0.0003
7.0	6.2	86.111	0.0238	−0.0726	−0.0488	−0.0003
Average..................................					−0.0485	

nicotine iron protoporphyrin in *alcoholic* solution give the expected value of 1 for n, repeated titrations of nicotine protoporphyrin in *aqueous* solution give clear evidence that n is 2. Since but 1 equivalent of reducing agent per gm. atom of iron is necessary to effect complete reduction, and since the spectral changes closely resemble those of other systems, it is proper to assume that a ferrous-ferric change is being followed. We must conclude that we have to deal with dimers containing 2 atoms of iron per molecule. That *both* oxidant and reductant are dimers is shown

by the symmetry of the titration curves.   The distinction between the conduct of nicotine iron protoporphyrin in aqueous and in aqueous alcoholic solution must be kept in mind in what is to follow.

Effect of pH—The examination of the effect of pH proves more difficult with the systems under consideration than with ordinary oxidation-reduction systems.   Frequently, so much coordinating base was added as to render calculation of the change in acid-base

TABLE VI

Pyridine Iron Hematoporphyrin System

Titration of pyridine ferrihematoporphyrin with reduced phthiocol at constant pH and constant pyridine concentration.   Demonstration that $n = 1$.   Pyridine $= 1.13$ M; total pigment $= 1.11 \times 10^{-4}$ M; phthiocol $\doteqdot$ 1.5×10^{-3} N; pH of phosphate buffer $+$ pyridine $= 11.75$; $\mu = 0.50$; $d = +0.20$ ml.; temperature $= 30°$; 100 per cent reduction at $y = 3.4$ ml.

v	$v - d$	Reduction	$0.06011 \times \log \frac{S_r}{S_o}$	E_h	E_b	Deviation from average
ml.	*ml.*	*per cent*		*volt*	*volt*	*volt*
0.5	0.3	9.375	-0.0592	-0.0570	-0.1162	-0.0005
0.9	0.7	21.875	-0.0332	-0.0800	-0.1132	0.0025
1.3	1.1	34.375	-0.0169	-0.0976	-0.1145	0.0012
1.6	1.4	43.750	-0.0066	-0.1093	-0.1159	-0.0013
2.0	1.8	56.250	-0.0066	-0.1239	-0.1173	0.0004
2.3	2.1	65.625	0.0169	-0.1348	-0.1179	-0.0022
2.6	2.4	75.000	0.0287	-0.1462	-0.1175	-0.0018
2.9	2.7	84.375	0.0441	-0.1568	-0.1127	0.0029
3.2	3.0	93.750	0.0707	-0.1719	(-0.1012)	0.0062
Average					-0.1157	

properties of the buffer systems uncertain.   Further, the effect of the nitrogenous base addition upon the systems could not be measured by the hydrogen electrode, for the latter was found unreliable in the presence of such materials.   Recourse was had to the glass electrode, although the high alkalinity of our solutions impaired the accuracy of this device.

The method of operation, the general nature of the results, and the limitations of interpretation placed upon "pH" values have been briefly outlined in Paper I (8).
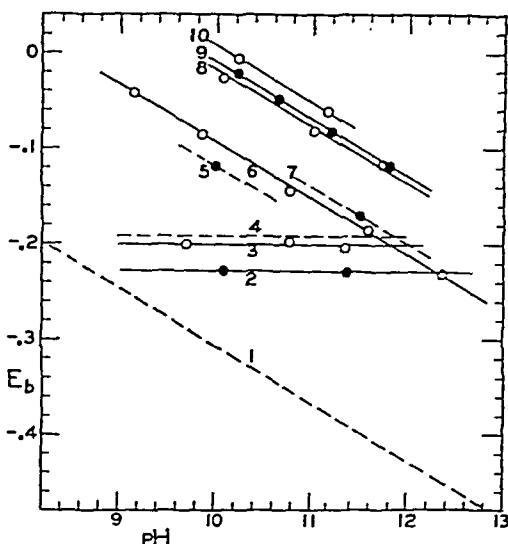
FIG. 1. Relation of E_b to pH. E_b is the potential at 50 per cent reduction and constant concentration of coordinating base. Curve 1, estimated for iron protoporphyrin (see Barron (1)); Curve 2, cyanide iron mesoporphyrin in water solution, total metalloporphyrin 1.25×10^{-4} M, cyanide 8.15×10^{-3} M, $E_b = -0.2291 \pm 0.0005$ volt; Curve 3, cyanide iron hematoporphyrin in water solution, total metalloporphyrin 1.72×10^{-4} M, cyanide 0.022 to 0.0082 M, $E_b = -0.200 \pm 0.004$ volt; Curve 4, cyanide iron protoporphyrin in water solution (see Barron (1)); Curve 5, α-picoline iron hematoporphyrin in water solution, total metalloporphyrin 1.32×10^{-4} M, α-picoline 9.3×10^{-1} M, a single titration at pH 10.05, $\mu = 0.1$, $E_b = -0.1224$ volt; Curve 6, pyridine iron mesoporphyrin in 47.5 per cent ethanol solution, total metalloporphyrin 1.47×10^{-4} M, pyridine 0.456 M, $\mu = 0.05$; Curve 7, nicotine iron mesoporphyrin in 47.5 per cent ethanol solution, total metalloporphyrin 1.47×10^{-4} M, nicotine 2.41×10^{-1} M, a single titration at pH 11.5, $\mu = 0.19$, $E_b = -0.1761$ volt; Curve 8, pyridine iron hematoporphyrin in water solution, total metalloporphyrin 1.1×10^{-4} M, pyridine 1.13 M, $E_b = 0.5856 - 0.0601$ pH, maximum deviation -0.009 volt; Curve 9, nicotine iron protoporphyrin in water solution, $n = 2$, total metalloporphyrin 4.76×10^{-4} M, nicotine 0.29 M, $\mu = 0.2$, $E_b = 0.5909 - 0.0601$ pH, maximum deviation -0.0012 volt; Curve 10, nicotine iron protoporphyrin in 47.5 per cent ethanol solution, total metalloporphyrin 1.44×10^{-4} M, nicotine 0.353 M, $\mu = 0.05$, $E_b = 0.6075 - 0.0601$ pH, maximum deviation 0.0002 volt.

Fig. 1 summarizes the data on the relation of "pH" to the characteristic potentials of the several systems each at constant

concentration of total base, S_b, approximately constant ionic strength, constant temperature (30°), and 50 per cent, reduction. Other details are given in the legend of Fig. 1.

Consideration of the contrast between the cyanide compounds, the potentials of which are invariant with pH, and the other systems which exhibit the relation $-\Delta E_h/\Delta \text{ pH} = 0.0601$ will be postponed to the section dealing with a postulated structural basis for the difference.

Potentiometric Measurements of Association—

$$O + q\text{B} \rightleftharpoons O\text{B}_q \text{ and } R + r\text{B} \rightleftharpoons R\text{B}_r$$
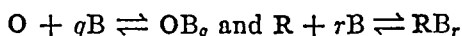
The potentiometric measurements of association were limited in scope: first, by reason of the instability of potential at low base concentration, a situation that approaches the case of a metalloporphyrin in the absence of base and that has been mentioned already; second, by the difficulty of resolving the effect of added base alone when the concentration of base becomes so high as to affect seriously other factors that must be controlled. Nevertheless, significant data were obtained in favorable cases. Diagrams are used to present the more important data, since they lend themselves to the proximate analysis outlined in Paper I of this series.

Fig. 2 gives an excellent example of the obscuring character of an association curve plotted with S_b as abscissa. The theoretical curve at the left has been drawn with constants calculated as described below but it is difficult to see any of the characteristics of the system. On the other hand the curve with log S_b as abscissa is revealing.

The data were submitted to the graphical analysis of Proposition V properly adjusted to the evidence of Table V that $n = 2$ and $p = 1$. Accordingly the proximate placement of the central portion of the curve was made with log $(S_b - 2S)$ as abscissa (case of $Z = 2$). A supplementary curve with log $(S_b - S)$ as abscissa was used to place the lower unique point where $K_R \rightleftharpoons$ [B]^4. The final theoretical curve of Fig. 2 has been drawn with log S as abscissa, after slight empirical adjustments of the constants to obtain a good fit. The following deductions are drawn from the *prima facie* evidence by objective methods of analysis.

By Proposition III nicotine coordinates more readily with the

reductant than with the oxidant. Since Table V shows that
$n = 2$ and $p = 1$, a proper use of Proposition VI indicates that
$r = 4$. The graphical analysis gives $K_R = 1.2 \times 10^{-10}$ and
$E_0' = -0.2570$ (at 50 per cent reduction $E_0' = -0.2381$). The
departure of the maximal slope from 0.12 is greater than would be
expected from the first order approximation of the ratio of K_o to
K_R were the same number of molecules of nicotine to combine
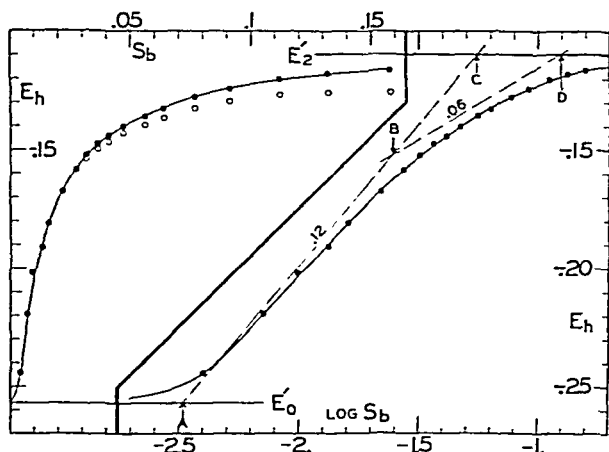
FIG. 2. Iron protoporphyrin IX + nicotine; aqueous solution; 30°.
O observed; ● corrected for pH change; S (on Fe basis) 2×10^{-4} M; 81
per cent reduced; pH = 11.1; $\mu = 0.35$; aqueous phosphate buffer. The
curves are calculated with $\frac{[R_2][B]^4}{[B_4 R_2]} = K_R$, $\frac{[B_2 O_2][B]^2}{[B_4 O_2]} = K_{O_1}$, $\frac{[O_2][B]^2}{[B_2 O_2]} =$
K_{O_2}, $K_R = 1.2 \times 10^{-10}$, $0.25 \log K_R = -2.48$ (see Point A), $K_{O_2} = 6.05 \times$
10^{-4}, $0.5 \log K_{O_2} = -1.609$ (see Point B), $0.25 \log K_{O_1} K_{O_2} = -0.256$ (see
Point C), $K_{O_1} = 1.56 \times 10^{-2}$, $0.5 \log K_{O_1} = -0.903$ (see Point D), $E'_0 =$
-0.2570 volt, $E'_2 = -0.1099$ volt.

with the oxidant and reductant. Furthermore, the curvature
of the upper part is not as abrupt as would then be expected and
it gives clear evidence of tending toward a "0.06 slope" for some
distance. These features suggest either $r - q = 2$ or stepwise
association involving a $K_o[B]^2$ term as well as a $[B]^4$ term. Under
the influence of the last the curve would finally follow a limiting
asymptote E_2. To give a fair representation of a distinct possi-

bility the theoretical curve has been drawn with the assumption
of the two successive processes:

$$O_2 + 2B \rightleftharpoons B_2O_2$$

$$B_2O_2 + 2B \rightleftharpoons B_4O_2$$
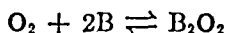
A difficulty ensuing from the last assumption will be noted in the
discussion.

Fig. 3 compares the iron mesoporphyrin and iron protopor-
phyrin systems associating with nicotine in 47.5 per cent alcoholic
water solutions.   By Proposition III the change of potential
shows $K_O > K_R$.   Sufficient of the whole course of association is
revealed to indicate that K_O is so much greater than K_R that
Proposition VI may be applied to the estimate of r.   Accordingly
$r = 2$.   That q is greater than 0 is evident by the departure from
the maximal slope as log S_b increases (Proposition VIII).   Obvi-
ously the data do not extend far enough to decide between $q = 1$
and $q = 2$.   The data for the meso system seem to fit the assump-
tion that $q = 1$ and accordingly the theoretical curve has been
drawn on that basis but without the intention of declaring a final
conclusion.   The data for the upper part of the association curve
of the proto system are in sufficient disorder to permit either
assumption.   This is indicated in Fig. 3.

It is evident that very much larger concentrations of associating
ase would be required to obtain certain proof that the curves of
ig. 3 approach limiting asymptotes having a "0.06 slope."
Should this slope be established, it would indicate $q = 1$ by
Proposition IX.   Nevertheless, a purely mathematical treatment
may proceed tentatively on the assumption of $q = 1$ within the
limits of the experimental conditions.

Fig. 4 presents a set of data for the association of the iron
protoporphyrins with cyanide ion.   We may conclude from the
data that $K_O > K_R$ but that the ratio of these constants is too
low to permit the use of those approximations that facilitate
graphical analysis.   The data indicate that within the range of
cyanide concentration investigated $q = r$.   Hogness, Zschiele,
Sidwell, and Barron (9) have given spectrophotometric evidence
that in this case the oxidant participates in the process

$$O_2(\bar{O}H)_2 + 4\bar{C}N \rightleftharpoons 2O(\bar{C}N)_2 + 2\bar{O}H$$
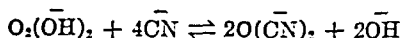
If this equilibrium be incorporated in the electrode equation, the latter will have a complexity that renders very difficult any
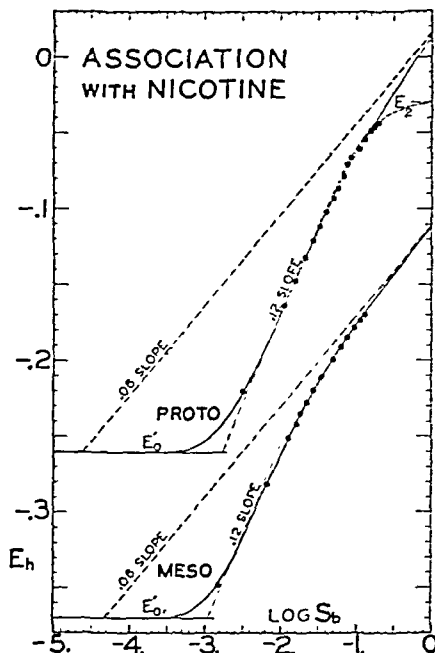
FIG. 3. Relation of potential to log [nicotine]; aqueous alcoholic solution; 30°. The upper curve represents iron protoporphyrin + nicotine in 47.5 per cent ethanol-water solution; phosphate buffer; "pH" = 11.16: $\mu = 0.05$; $S = 1.4 \times 10^{-4}$ M; per cent reduction = 23.5. The curve for $r = 2, q = 1$ is drawn with $K_R = 3 \times 10^{-6}$, $K_O = 1.2 \times 10^{-1}$, $E'_0 = -0.2605$ volt; the curve for $r = 2, q = 2$ is drawn with $K_R = 3 \times 10^{-6}$, $K_O = 2.2 \times 10^{-2}$, $E'_0 = -0.2605$ volt. The lower curve represents iron mesoporphyrin + nicotine in 47.5 per cent ethanol-water solution; phosphate buffer; "pH" = 11.10; $\mu = 0.2$; $S = 1.4 \times 10^{-4}$ M; per cent reduction = 46. The curve for $r = 2, q = 1$ is drawn with $K_R = 1.2 \times 10^{-6}$, $K_O = 2.5 \times 10^{-2}$, $E'_0 = -0.3701$ volt.

graphical analysis. This will be especially true if a similar change in state of aggregation fails to occur in the formation of the cyanide ferroprotoporphyrin. The analysis given in Fig. 4 is based on

the assumption that all four metalloporphyrins are monomeric. It is not maintained, however, that an equally satisfying "fit" may not be possible on some other basis.

Spectrophotometric Measurements of Association—In the study of the coordination of nitrogenous bases with ferro- and ferriporphyrins, the limitations imposed by solubility and other physical properties have forced us to arrive at the characteristics of the equilibria from the consideration of isolated fragments of data. The greater simplicity of solutions containing nitrogenous base and iron porphyrin complexes in but one stage of oxidation would
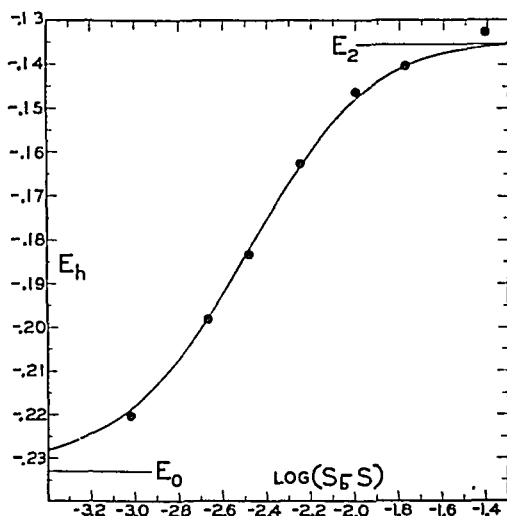
FIG. 4. Iron protoporphyrin + cyanide in phosphate buffer. Relation of potential to log $(S_b - S)$; pH $= 11.1$; $\mu = 0.4$; initial $S = 2.4 \times 10^{-4}$ M; per cent reduction $= ca.$ 13. The curve for $r = 2$, $q = 2$ is drawn with $K_R = 1.5 \times 10^{-6}$, $Ko = 6.39 \times 10^{-5}$, $E'_0 = -0.233$ volt.

appear to make spectrophotometric experiments less ambiguous. And yet the possibility that more than one absorbing species will make invalid the ordinary interpretation and the simpler mathematical analysis of photometric data is disturbing. What proved to be more aggravating in the present instance was the inadequacy of the available instrumental means of determining those slight changes of transmittance of critically important systems that are necessary to the complete elucidation of the equilibria.

The instrument of use was a Bausch and Lomb spectrophotometer. The light housing was replaced by one made according

to the specifications of McNicholas (10). The wave-length drum was calibrated by use of helium and hydrogen Plücker tubes. In the later measurements the absorption tubes were jacketed and surrounded with water circulated from a constant temperature bath.

The following is one item from the spectrophotometric evidence of the reversibility of the association between metalloporphyrin and base. Two ferriprotoporphyrin solutions were prepared with
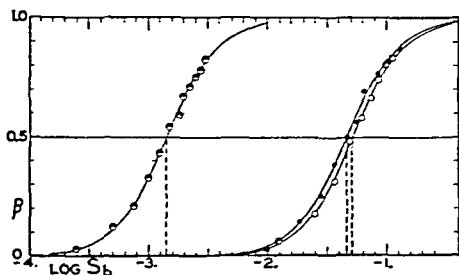
FIG. 5. Spectrophotometric association curves. Ordinate β = [base metalloporphyrin]/S; abscissa, log molar concentration of nicotine. ◐ *ferromesoporphyrin-nicotine in 47.5 per cent ethanol solution, 0.01 N in* NaOH; $S = 6 \times 10^{-6}$ M; $t = 23°$; 10 cm. column; $\lambda = 545$ mμ. The curve is drawn for $\dfrac{[R_m][B]^2}{[B_2 R_m]} = K_R$, $K_R = 2 \times 10^{-6}$, 0.5 log $K_R = -2.85$. ● ferri-mesoporphyrin + nicotine in *aqueous* phosphate buffer solution; pH = 10.8; $S = 2.61 \times 10^{-5}$ M; $t = 23°$; 10 cm. column; $\lambda = 580$ mμ. The curve is drawn for $\dfrac{[O_n][B]^2}{[B_2 O_n]} = K_O$, $K_O = 2.13 \times 10^{-3}$, 0.5 log $K_O = -1.34$. ○ ferri-protoporphyrin + nicotine in *aqueous* phosphate buffer solution; pH = 11.1; $S = 6.08 \times 10^{-5}$ M; $t = 23°$; 5 cm. column; $\lambda = 580$ mμ. The curve is drawn for $\dfrac{[O_n][B]^2}{[B_2 O_n]} = K_O$, $K_O = 2.6 \times 10^{-3}$, 0.5 log $K_O = -1.29$.

intermediate concentrations of nicotine such as to give considerable differences in spectral absorption. On dilution of the solution stronger in nicotine to the nicotine concentration of the other and maintenance of the total pigment concentration, the spectral absorptions adjusted to equality.

Paper I (8) describes the procedure for the spectrophotometric study of association.

Fig. 5 shows the data for nicotine ferromesoporphyrin in 47.5

per cent alcohol. The data were transferred to a rectified line by Proposition XIV and the calculated constants were then employed in constructing the type curve for the addition of 2 molecules of nicotine to 1 of ferromesoporphyrin in state of aggregation m. Since the potentiometric data have indicated that $m = n = p = 1$, in aqueous *alcoholic* solution, this is a confirmation of the potentiometric evidence that $r = 2$. The constant is in substantial harmony with $K_R = 3 \times 10^{-6}$, the approximate, graphical estimate on the basis of which the curve of Fig. 3 was constructed.

Fig. 5 also summarizes data for the association of nicotine with ferrimesoporphyrin and ferriprotoporphyrin in *aqueous* phosphate buffer solution. In each case the rectified curves indicated the process $O_n + 2B \rightleftharpoons B_2O_n$ and the type, sigmoid curves have been constructed accordingly. These cases will be discussed later.

Attempts to complete the coordination picture with spectrophotometric studies of ferroprotoporphyrin in *aqueous* solution and ferrimesoporphyrin in *alcoholic* solution with nicotine were unavailing. Reproducible curves in the former case could not be obtained. The readings in solutions of low nicotine content were particularly unstable. The visible spectra of ferrimesoporphyrin and of its nicotine and pyridine complexes in alcoholic solution were too similar to permit measurements of the required precision with the instrument at hand. This was found true for alcoholic solutions of all the ferriporphyrins and their nicotine and pyridine complexes. The complexes of iron hematoporphyrins appeared to be attractive materials for a complete study of the coordination reactions, since the ferrous species were sufficiently soluble and the spectra of the ferric forms were dissimilar enough in aqueous solution, but a light scattering impurity in our preparations prevented significant measurements.

Without dwelling further on such difficulties, we may say that when the spectrophotometric studies are considered in conjunction with the potentiometric association experiments something of interest will appear.

Effect of Ionic Strength—We have preferred to regard the ionic strength effect to be of secondary importance pending the development of other features and provided the variation of the ionic strength of the buffer solutions be not great enough to

affect seriously the study of variables having first order importance. For the preliminary study of the ionic strength effect the pyridine iron mesoporphyrin system in alcoholic solution was chosen. While not ideal, it possesses less unfavorable properties than the others. Fixing the total pyridine, the degree of reduction, and the ratio of buffer salt to buffer acid, we added known quantities of potassium chloride to the system. This will change both the ionic strength and the "pH numbers." A correction for the latter can be made from the known response of the system to changes of "pH." Using the value 0.611 for the dielectric constant obtained from interpolation of Wyman's (11) data for
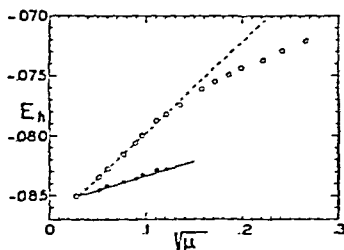
Fig. 6. Relation of ionic strength and potential in the pyridine iron mesoporphyrin system. O represents experimental data, ● experimental data at the higher dilutions corrected for pH changes. The slope of the latter curve is 0.0240. Solvent, borate buffer 47.5 per cent in ethanol; initial pH 10.21; total metalloporphyrin 1.4×10^{-4} M; per cent reduction 25.

similar water-alcohol mixtures we can deduce from the limiting equation of Debye and Hückel the relation

$$\frac{dE_h}{d\sqrt{\mu}} = 0.0518(z_r^2 - z_o^2)$$

where z_r and z_o are the charges of reductant and oxidant respectively. The observed data are plotted in Fig. 6 together with the same data corrected for the estimated changes of "pH" that accompany the addition of KCl. Assuming that the lower measurements are at ionic strengths low enough to permit their use in guiding the placement of a limiting line, we may make the slope thereof: $dE_h/d\sqrt{\mu} = 0.024$. This corresponds to no integral values of z_r and z_o. Discussion will be postponed.

DISCUSSION

The commonly accepted structure of ferriprotoporphyrin chloride in the solid state is the following.

$$
\left[
\begin{array}{c}
\text{CH}_3\text{—C——C—CH}=\text{CH}_2 \\
\text{(porphyrin ring with Fe}^{+++}\text{)}
\end{array}
\right]^{+} \quad \text{Cl}^{-}
$$

CH₃—C———C—CH=CH₂
        C   C
      CH   N⁻   CH
CH₂=CH—C—C   C—C—CH₃
          N   Fe⁺⁺⁺   N
CH₃—C—C   C—C—C₂H₄COOH
        HC   N⁻   CH
          C   C
CH₃—C====C—C₂H₄COOH
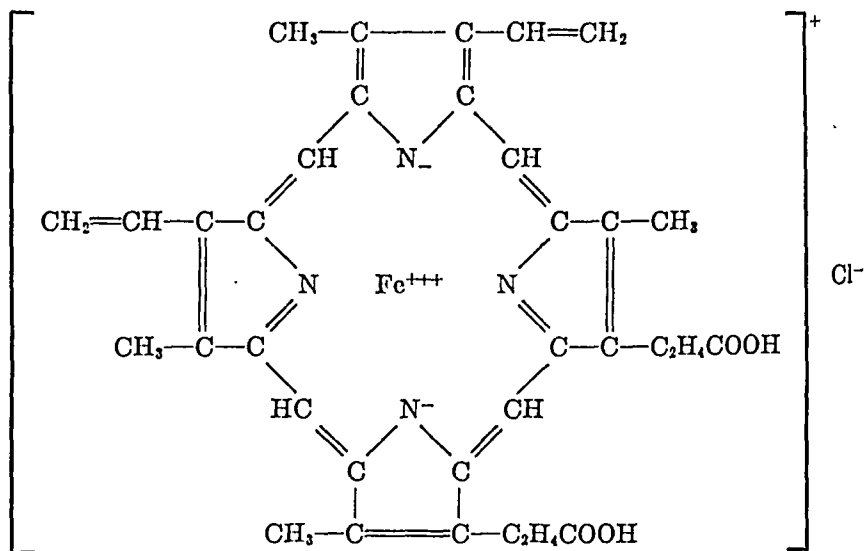
This is inadequate to account for the behavior of the compound in solution.

Since the evidence is that the oxidant is concerned with a hydroxyl, we may say now that not only ferriprotoporphyrin but also ferrimesoporphyrin and ferrihematoporphyrin appear to associate with 1 hydroxyl ion per iron atom in alkaline aqueous and aqueous alcoholic solution. In the case of dimerization, 2 hydroxyl ions associate per molecule.

It will add to the clarity of a schematic representation if we introduce a postulate, of which we have no evidence; namely, that when there are lacking other associating molecules, the usual coordination number, 6, of iron is satisfied by the entrance of water.[1] Then four coordination positions are occupied by pyrryl nitrogens, one by hydroxyl ion, and one by a water molecule as represented below.[2]

---

[1] The evidence given by Katz (12) that "α-Hämine" and "β-Hämine" orm no definite hydrates refers to the crystal states as does Richter's 13) evidence of other additions.

[2] The scheme will require modification if data now accumulating should demonstrate that the polymerization noted in several of the ferriporphyrins

$$\left[\begin{matrix} & N^- & \\ & \overline{OH} & \\ N & Fe^{+++} & N \\ H_2O & & \\ & N^- & \end{matrix}\right]_n + nH^+ + ne \rightleftharpoons \left[\begin{matrix} & N^- & \\ & H_2O & \\ N & Fe^{++} & N \\ H_2O & & \\ & N^- & \end{matrix}\right]_n$$
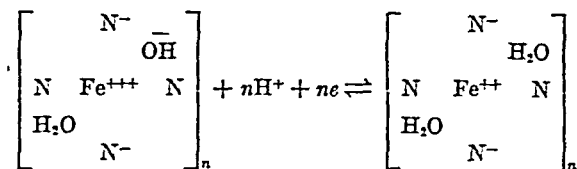
or with abbreviated symbols

$$(O\overline{OH})_n + nH^+ + ne \rightleftharpoons R_n$$
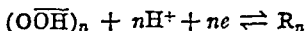
The essential points in the following theory are (1) that all associating bases can compete easily with the 2 water molecules for position in the coordination shell of a ferroporphyrin; (2) that neutral associating bases can compete successfully but less easily with the 1 molecule of water in the coordination shell of a ferriporphyrin; (3) that neutral associating bases cannot compete with the hydroxyl ion in the coordination shell of a ferriporphyrin except possibly at very high concentrations of base; (4) that cyanide ions can compete easily with the hydroxyl ion in the coordination shell of a ferriporphyrin.

Let us describe model relations by means of monomeric components and provide a basis for discussion by postulating the following equilibria.

$$\frac{[R][B]^2}{[B_2R]} = K_R \quad (1) \qquad \qquad \frac{[O][B]^2}{[B_2O]} = K_{O_1} \quad (2)$$

$$\frac{[O\overline{OH}][H^+]}{[O]} = K_h \quad (3) \qquad \qquad \frac{[O\overline{OH}][B]}{[BO\overline{OH}]} = K_{O_2} \quad (4)$$

Note: Process $O\overline{OH} + 2B + H^+ \rightleftharpoons B_2O$ is accounted for by use of Equations 2 and 3.

$$S_r = [R] + [B_2R] \quad (5) \qquad S_o = [O] + [O\overline{OH}] + [B_2O] + [BO\overline{OH}] \quad (6)$$

is effected through bonds occupying coordination positions of the iron atoms. The spectra of the ferri- and ferroporphyrins in alkaline aqueous solution differ from those in aqueous alcoholic solution. The spectra of the nitrogenous base complexes, however, are unaffected by the change in solvent. Since such changes in spectra usually imply alterations in structure, it is not unreasonable to assume that in alcoholic solution the water of ferri- and ferroporphyrin has been partially or completely replaced by ethanol.

Whence

$$E_h = E_0 + 0.06 \log \frac{S_o}{S_r} + 0.06 \log \frac{K_{O_1} K_{O_2}}{K_R}$$

$$+ \, 0.06 \log \frac{[\mathrm{H^+}](K_R + [\mathrm{B}]^2)}{K_{O_1} K_{O_2}([\mathrm{H^+}] + K_h) + K_h K_{O_1}[\mathrm{B}] + K_{O_2}[\mathrm{B}]^2[\mathrm{H^+}]} \qquad (7)$$

The cases in which [H$^+$] and [B] are constant and S_o/S_r is varied have been discussed adequately so that we now may assume a constant degree of reduction. Also the experiments do not cover the direct test of Equation 3 so that we may assume [H$^+$] < K_h. Whence

$$E_h \propto 0.06 \log \frac{[\mathrm{H^+}](K_R + [\mathrm{B}]^2)}{K_{O_1} K_{O_2} K_h + K_h K_{O_1}[\mathrm{B}] + K_{O_2}[\mathrm{B}]^2[\mathrm{H^+}]} \qquad (8)$$

When [B] is 0

$$\frac{-dE_h}{d\,\mathrm{pH}} = 0.06 \qquad (9)$$

Equation 9 is supported by Barron's data and by the approximate estimates of our own.

In the case of the neutral bases such as pyridine and nicotine we may assume such lack of success in their competition with hydroxyl ion that usually the species B$_2$O may not form except at very high values of [B]. Whence

$$E_h \propto 0.06 \log \frac{[\mathrm{H^+}](K_R + [\mathrm{B}]^2)}{K_{O_2} + [\mathrm{B}]} \qquad (10)$$

Then, at any constant value of [B]

$$\frac{-dE_b}{d\,\mathrm{pH}} = 0.06 \qquad (11)$$

For confirmation of Equation 11 see Fig. 1. Equation 11 presumably will hold also for the dimeric iron protoporphyrin, since there will obtain an equation comparable to Equation 7 but with the coefficient 0.03 and the term [H$^+$]^2.

At constant degree of reduction and constant [H$^+$] the final trend of $dE_h/(d \log S_b)$ will depend on whether B$_2$O or BO$\overline{\mathrm{OH}}$ is the final form. If formation of the species B$_2$O is too difficult, $dE_h/(d \log S_b) = 0.06$ as exhibited.

In the cases of the cyanide systems we may assume such ease of competition against [$\overline{OH}$] that the process will be

$$O\overline{OH} + 2\overline{CN} + H^+ \rightleftharpoons O(\overline{CN})_2$$
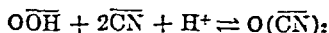
involving Equations 2 and 3 as well as Equation 1. Then

$$E_k \propto 0.06 \log \frac{[H^+](K_B + [B]^2)}{K_{O_1} K_k + [B]^2[H^+]}$$

If $[B]^2 > K_{O_1}K_{k_1}/[H^+]$, $\Delta E_k/\Delta$ pH $= 0$. This requires that, when [H$^+$] is very small, K_{O_1} be very small. While the evidence is that K_{O_1} is small, the final satisfaction of this argument must rest upon a direct estimate of K_{O_1} and this is not available.

There appears a conflict between the *prima facie* evidence of Fig. 2 and that of Fig. 5. The potentiometric evidence that more than 1 nicotine per atom of iron (2 nicotines per mole of dimer in water solution) adds to the oxidant is open to question because of the difficulty of correcting the data at high concentrations of nicotine to a common basis and because it is not certain that more than the estimated number of molecules of base may not add to reductant at very high concentrations of base. The spectral evidence is that 2 molecules of nicotine add per molecule but the spectral evidence will not distinguish the cases $O_2 + 2B \rightleftharpoons B_2O_2$ and $O + 2B \rightleftharpoons B_2O$. The spectral evidence is open to question because of the inherent instrumental and theoretical difficulties.

If the treatment accorded the data of Fig. 2 be correct, and the model of Equation 7 be retained and modified only to fit this case of dimers, the final dominance of the species B_4O_2 would lead to the relation $-dE_b/d$ pH $= 0$. Of this there is no sign. If, however, the species of oxidant containing 4 molecules of nicotine retain 1 hydroxyl ion per iron atom, the relation $-dE_b/d$ pH $= 0.06$ will be satisfied but the postulated species would violate the usual "rule of 6" for the coordination number of iron.

The data of Fig. 5 on the association of nicotine with ferrimesoporphyrin in alkaline aqueous solution permit two interpretations. Either the metalloporphyrin is monomeric and associates with 2 nicotine molecules in the range of nicotine concentration studied or it is dimeric and adds 1 nicotine molecule

per iron atom.   Potentiometric data which would lead to a decision here were unobtainable by reason of the very low solubilities of ferromesoporphyrin and nicotine ferromesoporphyrin already mentioned.   The very similar spectral changes observed when nicotine is added to either ferrimesoporphyrin or ferriprotoporphyrin and the close proximity of the two curves of Fig. 5 suggest that the two metalloporphyrins and their complexes with nicotine are highly similar in structure.   By this argument ferrimesoporphyrin would be a dimer adding 1 molecule of nicotine per iron atom.

Even a superficial examination of the equations constructed on simplifying assumptions and without the inclusion of possible but unidentified species will show that several important decisions can be made only when consistency is established among the numerical values of several constants.   Since the systems so far examined have properties that have precluded the evaluation of some of the constants, there is no point in further discussion at this time.   Systems better suited to the examination of doubtful relations should be sought.

What seems to remain clear in addition to the relations developed in the experimental section is that competition between coordinating bases and hydroxyl ions for the bonds of the coordination shell plays a decisive rôle.

Although dimerization of Werner complexes of transition group elements has been observed in several instances (14, 15), the structures proposed for these do not appear applicable to our systems.   Our data on the dimerized systems reveal the two coordination centers to be intimately linked energetically.   The data of Table V indicate the species in which 1 iron ion is ferric and 1 is ferrous to be quite unstable with respect to the species in which both iron ions are ferric or ferrous.   Similarly, there is no evidence in the data of Fig. 5 or of Fig. 2 for the stepwise addition of the first 2 nicotine molecules to the dimer.   This interdependence of the iron atoms suggests a hydroxyl bridge between the coordination centers such as Gaines, Hammett, and Walden (15) found in the complexes of iron and phenanthroline.   If this holds, we are at a loss to explain the extreme sensitivity of dimerization to apparently trivial alterations in the substitutions on the pyrryl rings.   The nicotine iron coproporphyrin system exhibits no polymerization under the same con-

ditions in which the nicotine iron protoporphyrin system is dimeric. Further, Barron (1) reports the pyridine iron protoporphyrin system to be completely dimeric at pH 11.98, whereas the pyridine iron hematoporphyrin system appears completely monomeric at pH 11.75 (Table VI). The well known tendency toward polymerization of compounds carrying aromatic vinyl groups (see for example (16)) suggests the vinyl side chains of protoporphyrin as the site of the dimerizing bond. This conflicts, however, with the interpretation we have given for the spectrophotometric association data on the nicotine ferrimesoporphyrin system. The theory fails to account for the contrasting behavior of pyridine iron protoporphyrin with α-picoline iron protoporphyrin (1).

The evidence for the state of aggregation of the iron porphyrins in the absence of coordinating base is conflicting. The data on Fig. 4 have been analyzed with fair success on the assumption that ferric protoporphyrin is dimeric. This is in accord with the report of Hogness et al. (9) on the association of ferriprotoporphyrin with cyanide ion but at odds with our own analysis of the potentiometric data on the system. The theoretical curve of Fig. 2 was constructed on the assumption that both ferro- and ferriprotoporphyrin existed as dimers in alkaline aqueous solution.

If the iron protoporphyrin system in the absence of coordinating base is such that the oxidant is a dimer and the reductant a monomer, the potential should be a unique function of the total concentration of the system. Conant and Tongberg (17) reported the potential to become more negative with increase of the system's concentration, which is the direction predicted on the above assumption. On the other hand Barron reports a change of potential with dilution which is opposite in sign to that observed by Conant and Tongberg and which is greater than would be expected for a monomeric reductant and dimeric oxidant. To complicate matters Barron's data for individual titrations are fairly well described by the equation derived on the assumption that both oxidant and reductant are monomeric. We regret that our own attempts to deal with this system have not yielded reliable data, for the characterization of the metalloporphyrin systems in the absence of coordinating base is essential to a complete understanding of the subject as a whole.

It remains to place Taylor's (3) results with cobalt and manganese mesoporphyrins in harmony with the structural hypothesis here outlined.   As Taylor has emphasized, the range of experiment was limited by the properties of the components of his systems.   But to be within the realm of the established relations we need only postulate that manganic mesoporphyrin, like the reductants of the iron porphyrins, either does not coordinate with hydroxyl ion or does so only at pH values greater than those used by Taylor.   This should remove the pH effect and Taylor found none.   The absence of hydroxyl ion should also result in charges leading to a greater ionic strength effect and this is what Taylor found.

The work of other investigators with other methods lends support to the postulated structures.   The center of coordination of the postulated compounds bears no net charge.   Thus the metalloporphyrins formed from porphyrins having no carboxyl group should be electrically neutral in alkaline solution.   Haurowitz (18) reports that the dimethyl ester of ferrimesoporphyrin IX in alkaline alcohol-water mixtures does not migrate in the electric field, while on acidification of the solution the pigment migrates as a cation.   This is consistent with the formulation

$$\begin{bmatrix} & N^- & \\ & \overline{O}H & \\ N & Fe^{+++} & N \\ H_2O & & \\ & N^- & \end{bmatrix}^0 + H^+ \longrightarrow \begin{bmatrix} & N^- & \\ & H_2O & \\ N & Fe^{+++} & N \\ H_2O & & \\ & N^- & \end{bmatrix}^+$$
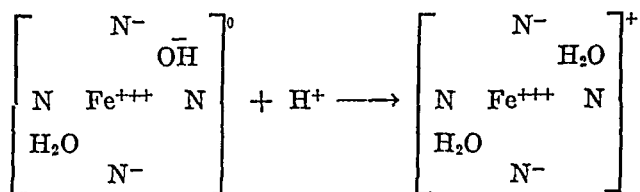
wherein *sources* of charges are indicated.   Haurowitz observed the cyanide compound to migrate as an anion.   This also is consistent with our formulation.

To form the disodium salt of ferriprotoporphyrin from the ferriprotoporphyrin chloride our postulate requires 3 equivalents of alkali per gm. atom of iron, as illustrated below.

$$\begin{bmatrix} & N^- & \\ N & Fe^{+++} & N \\ & N^- & \end{bmatrix}^+ \begin{matrix} \\ \overline{C}l \\ COOH \\ COOH \end{matrix} + 3\overline{O}H \longrightarrow \begin{bmatrix} & N^- & \\ & \overline{O}H & \\ N & Fe^{+++} & N \\ H_2O & & \\ & N^- & \end{bmatrix} \begin{matrix} \\ COO^- \\ COO^- \end{matrix} + \overline{C}l + H_2O$$
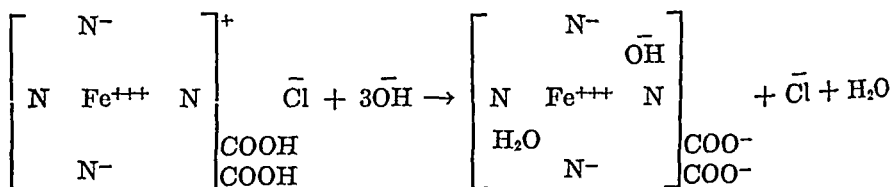
Morrison and Williams (19) reported titration curves which were complicated by the presence of a solid phase but in which 3 equivalents of alkali are required before the final "break."

In our scheme the oxidant and the reductant of pyridine iron mesoporphyrin bear the same net charges, namely two negative, accounted for by the ions of the carboxyl groups. Therefore, in the simplified theory of Debye and Hückel an "ionic strength effect" of the type noted in the experiment recorded by Fig. 6 should be nil. After correction of the data for pH changes there is a residual effect ascribable to change in ionic strength. It would be interesting to know whether this small residual effect is due to a small proportion of the species B_2O of net charge $z_o = 1$ $(-)$. Of course any simple argument in this field is predicated on the assumption that no allowance be made for the spatial distribution of charges and this may play a rôle in molecules of the kind under consideration.

SUMMARY

By potentiometric and spectrophotometric measurements data were obtained for the following.

1 equivalent per iron atom is concerned in the reduction of ferrimesoporphyrin, ferriprotoporphyrin, and ferrihematoporphyrin and in the presence of nicotine, pyridine, α-picoline, or cyanide. Oxidant and reductant of the nicotine iron protoporphyrin system are dimers in aqueous solution, monomers in 47 per cent aqueous alcohol within the range of pH used.

Measurements of the change of potential with increase of concentration of coordinating base, all other conditions being constant, have shown in all the cases that the ferroporphyrins coordinate better with bases than do the ferriporphyrins and that 2 molecules of base per iron atom coordinate with ferroporphyrins. The evidence that coordination occurs with ferriporphyrins is definite but whether 1 or 2 molecules of pyridine, nicotine, or α-picoline coordinate per iron atom is uncertain.

At constant high concentration of associating base and constant degree of reduction $-\Delta E_h/\Delta$ pH $= 0.06$ for all cases except cyanide, in which case the value is 0.

A structural hypothesis that accounts for the main features is that the ferriporphyrins in alkaline solution are associated with 1 hydroxyl ion per iron atom, that neutral bases compete with difficulty, if at all, with this hydroxyl ion for position in the coordination grouping, while the negatively charged cyanide ions compete successfully.

Owing to limitations imposed by the physical properties of the systems examined, certain quantitative relations flowing from the formulation of this theory have not been determined but their natures have been indicated.

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METALLOPORPHYRINS

IV. COORDINATION OF IRON COPRO- AND ETIOPORPHYRINS WITH NITROGENOUS BASES*

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Investigations by Barron (1), Clark, Taylor, Davies, and Vestling (2), Taylor (3), and Davies (4) have outlined several features of the coordination of metalloporphyrins with nitrogenous bases. There will now be reported potentiometric measurements with systems containing iron coproporphyrin, the properties of which have proved to be revealing, and also a few measurements with iron etioporphyrin, sufficient to check a point of some significance.

To establish a basis of comparison with the systems previously discussed the coordinating substances used were pyridine, nicotine, and cyanide. For the investigation reported here coproporphyrin I (1,3,5,7-tetramethyl-2,4,6,8-tetrapropionic acid porphine) and etioporphyrin I (1,3,5,7-tetramethyl-2,4,6,8-tetraethyl porphine) were synthesized. It is now possible to compare a series of systems in which the iron compounds of five different porphyrin structures are found. These are protoporphyrin IX, hematoporphyrin IX, mesoporphyrin IX, etioporphyrin I, and coproporphyrin I.

In addition, a preliminary study was made of the reversibility of the reaction
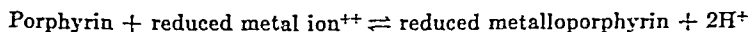
$$\text{Porphyrin} + \text{reduced metal ion}^{++} \rightleftharpoons \text{reduced metalloporphyrin} + 2\text{H}^+$$

in glacial acetic acid solution.

Preparations

Coproporphyrin I—The methods used were essentially those of Hans Fischer and coworkers (5). The procedure of Corwin and Quattlebaum (6) was used in the preparation of the starting material, 2,4-dimethyl-3,5-dicarbethoxypyrrole. Several modifications to be described were introduced in later steps which offered difficulty.

2,4-Dimethyl-3-carboxy-5-carbethoxypyrrole was obtained in 60 to 80 per cent yield from the 3-carbethoxy compound by treatment with concentrated sulfuric acid at 30°. Decarboxylation in glycerol (7) yielded 60 to 70 per cent of the next desired product, *2,4-dimethyl-5-carbethoxypyrrole* (m.p. 124°, uncorrected).

The modified Gattermann aldehyde reaction as described by Corwin and Andrews (8) was used to prepare 2,4-dimethyl-3-formyl-5-carbethoxypyrrole. The recrystallized pyrryl aldehyde melted sharply at 144–145° (uncorrected), and the yields varied from 60 to 77 per cent.

2,4-Dimethyl-3-formyl-5-carbethoxypyrrole was condensed with malonic acid in the presence of freshly distilled aniline (9) to give 60 to 80 per cent of *2,4-dimethyl-3-acrylic acid-5-carbethoxypyrrole* (m.p. 236–239°, with decomposition).

Catalytic hydrogenation with palladium chloride on charcoal (2 atmospheres of hydrogen) was found to be the most satisfactory method of reducing the acrylic acid side chain to the propionic acid side chain. The reduction went to completion (90 per cent yields) in alcohol solution, giving pure white *2,4-dimethyl-3-propionic acid-5-carbethoxypyrrole* (m.p. 158–159°, uncorrected) after recrystallization in alcohol. The pyrryl propionic acid is much more soluble in alcohol than the pyrryl acrylic acid; 200 ml. of alcohol and 17 gm. of the pyrryl acrylic acid were used in one preparation.

2,4-Dimethyl-3-propionic acid pyrrole (cryptopyrrolecarboxylic acid) was prepared as outlined below (10). These steps and the ones to follow offered great difficulty and the yields were very low.

A typical saponification and decarboxylation were carried out as follows: 3.3 gm. of purified carbethoxycryptopyrrolecarboxylic acid were placed in a 50 ml. Erlenmeyer flask equipped with a reflux condenser, 2.5 gm. of sodium hydroxide were added, and 20 ml. of distilled water. The mixture was heated on the steam bath for 3 hours, at the end of which time a slight yellow color was
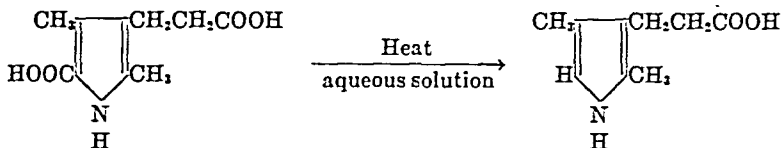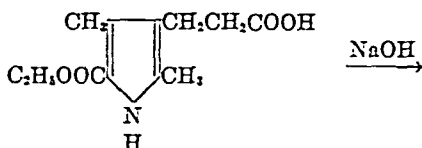
noticeable. The cooled solution of the disodium salt was filtered into a small beaker, and further cooled by the addition of solid carbon dioxide. Then, with rapid stirring, concentrated hydrochloric acid was added drop by drop until the system was just acid to Congo red. Since both the free acid and its decarboxylation product were extremely unstable in air, solid carbon dioxide was kept present in the system and a nitrogen cone was used to exclude air as completely as practicable. The free carboxycryptopyrrolecarboxylic acid was obtained in good yield as a light yellow, crystalline product. On exposure to air it turned deep red in a few hours. The crude acid was transferred rapidly under nitrogen to a small beaker, water (10 ml. per 2 gm. of pyrrole acid) was added, and the system heated to boiling under nitrogen. Decarboxylation took place smoothly and the solution became red in color. When evolution of carbon dioxide had ceased, the system was rapidly cooled, filtered, and the product dried *in vacuo* over solid potassium hydroxide for several hours. The cryptopyrrolecarboxylic acid crystallized in beautiful light yellow needles of sharp melting point, 145–147° (uncorrected); yields varied from 40 to 66.7 per cent. Several hours exposure to air resulted in the formation of a deep red, water-soluble oil.
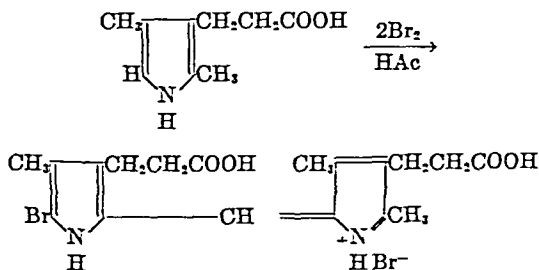
3′,4,5′-Trimethyl-3,4′-dipropionic acid-5-bromodipyrryl methene hydrobromide was prepared as described by Fischer and Fröwis (11), and fine red macroscopic crystals were obtained in 20 to 38 per cent yield (decomposition point, 220°, uncorrected). This compound was not analyzed, but was subjected to the final step in the synthesis.

Coproporphyrin I (1,3,5,7-tetramethyl-2,4,6,8-tetrapropionic acid porphine) (12) was obtained in poor yield by melting the methene hydrobromide in 3 times its weight of anhydrous succinic acid for 1 hour at 190°. Crude coproporphyrin I was obtained from the melt by grinding with dilute sodium hydroxide and filtering. The residue was extracted with strong hydrochloric acid until the extracts showed no more porphyrin spectrum. The hydrochloric acid and sodium hydroxide solutions were combined, sodium acetate was added nearly to saturation, and the pH adjusted to 4.6, with brom-cresol green as an indicator. On standing, a dark amorphous precipitate was obtained, which was centrifuged off, redissolved in dilute alkali, filtered, and reprecipitated several times. It was admittedly an impure product, but numerous attempts to crystallize the small amount at hand failed. From 0.8 gm. of methene hydrobromide about 0.25 gm. of total porphyrin was obtained. The bulk of the crude coproporphyrin I was made into the iron complex as described below, but a small amount was made into the tetramethyl ester for identification (9).

Coproporphyrin I tetramethyl ester was obtained in thick red-brown needles with a melting point of 250–253° (uncorrected, with decomposition). With the aid of a Zeiss hand spectroscope with calibrated wave-length scale and with the spectrophotometer the following absorption maxima were recorded:

Band I 492 to 508 mμ, Band II 524 to 538 mμ, Band III 560 to 570 mμ, Band IV 610 to 616 mμ. The order of intensity was Band I, II, III, IV.

Analysis of the recrystallized ester gave

$C_{40}H_{46}O_8N_4$. Calculated, C 67.60, H 6.47; found, C 67.04, H 6.95

The sample (10.341 mg.) left 1.33 per cent ash.

Etioporphyrin I (1,3,5,7-Tetramethyl-2,4,6,8-Tetraethyl Porphine)—The synthesis was carried out in this university by Walter Schlesinger for the most part according to the methods of Fischer

and Orth (5). The absorption maxima of the purified crystalline material in chloroform were as follows:

Band I 484 to 508 mμ, Band II 524 to 536 mμ, Band III 560 to 570 mμ, Band IV 610 to 618 mμ. The order of intensity was Band I, II, III, IV.

Analysis of the recrystallized material gave

$C_{22}H_{33}N_4$.   Calculated.   C 80.33, H 7.94, N 11.71
          Found.   " 80.99, " 8.17, " 11.83
                   " 79.83, " 7.93

Preparation of Iron Copro- and Iron Etioporphyrins—Preparative methods for the formation of metalloporphyrins from porphyrins and metal ions and for the splitting of metalloporphyrins for the purpose of recovering their porphyrin constituents are suggestive of the reversibility of the reaction

Porphyrin + metal ion^{++} $\rightleftharpoons$ reduced metalloporphyrin + 2H$^+$
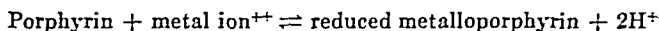
Fischer, Treibs, and Zeile (13) and Treibs (14) have investigated this reaction and have presented evidence for this postulate. Oxidation of the reduced metalloporphyrin and the precipitation of the chloride (of hemin, for example) favor a shift "to the right" with increased production of metalloporphyrin. When the metal ion enters, it replaces 2 protons in the tetrapyrryl ring (15). It seems probable that a competition between metal ions and protons might turn in favor of the protons in solutions of high proton activity so that this reaction would appear to go from "right to left."

Since very limited supplies of coproporphyrin I and etioporphyrin I were available, it was desired to have some surety in the rationale of the method for preparing the iron compounds. Accordingly, the following experiment was conducted with the more abundant mesoporphyrin IX.

Glacial acetic acid buffer solutions had been prepared according to the method of Conant and Chow (16). However, since most of their buffer systems contain nitrogenous materials which are prone to coordinate with metalloporphyrins, inorganic buffers were used. These included 1 M sodium acetate, $(pH)^{HAc} = 3.70$; 0.5 M sodium acetate, $(pH)^{HAc} = 3.60$; 0.1 M sodium acetate, $(pH)^{HAc} = 3.34$; 0.1 M magnesium acetate, $(pH)^{HAc} = 3.01$; 0.2 M barium acetate, $(pH)^{HAc} = 2.73$; saturated zinc acetate,

$(pH)^{HAc}$ = 2.13.   These were measured according to the method of Conant and Chow.

The absorption bands of the species concerned, as observed with a Zeiss hand spectroscope, are as follows:

Mesoporphyrin IX in Buffered Glacial Acetic Acid—Band I 525 mμ, Band II 545 to 560 mμ, Band III 570 mμ, Band IV 590 to 605 mμ (diffuse). The order of intensity was Band II, IV, III, I.

Ferrimesoporphyrin IX in Buffered Glacial Acetic Acid—Band I 530 to 542 mμ (diffuse), Band II 630 to 640 mμ (sharp).

Ferromesoporphyrin IX in Buffered Glacial Acetic Acid—Band I 545 to 560 mμ, Band II 555 to 565 mμ (the intensities and definition about equal).

None of these absorption bands shifted with change of $(pH)^{HAc}$.

No splitting of ferric mesoporphyrin was observed when concentrated hydrochloric acid (5 ml.) was added to 10 ml. of a saturated solution, confirming the well known fact that an oxidized metalloporphyrin is resistant to splitting.

In a solution of $(pH)^{HAc}$ 3.7 reduction (hydrogen and colloidal palladium) and reoxidation (air) of the complex were performed several times without splitting. By the sixth reduction distinct traces of mesoporphyrin were detected. Since completely reversible oxidation-reduction was not observed in this, the most "alkaline" buffer, it was impossible to investigate the system potentiometrically.

It was not found practicable, with the limited method used, to decide upon a value of $(pH)^{HAc}$ centering the region on one side of which splitting is definitely favored and on the other side of which the coordination is definitely favored. Nevertheless, it was found that the higher the acidity of the solution the more the splitting was favored.

Reduction of ferrimesoporphyrin in unbuffered glacial acetic acid led in a few moments to mesoporphyrin and to the inability to reform the ferrimesoporphyrin.

These suggestive results indicate a distinct advantage in giving the glacial acetic acid solution a high value of $(pH)^{HAc}$ by the addition of the strong base, sodium acetate. Accordingly this was done in the preparation of iron coproporphyrin and iron etioporphyrin.

The procedure adopted is illustrated by the following preparation of iron etioporphyrin I chloride: 200 mg. of recrystallized

etioporphyrin I were placed in 175 ml. of 1 M sodium acetate in glacial acetic acid, about 3 gm. of sodium chloride were added, and the mixture warmed to aid solution. The system was then transferred to a special apparatus which consisted essentially of two small Erlenmeyer flasks equipped with reflux condensers and inlet and outlet tubes in such a way that tank nitrogen could be passed through both flasks. The entire operation to be described was carried out under nitrogen, and the two flasks were so arranged that the contents of one could be poured into the other through its reflux condenser. Ferrous acetate was prepared by refluxing glacial acetic acid over iron filings for a few moments under nitrogen in one flask; then about 50 ml. of the cooled, solution of ferrous acetate were added through a glass wool plug to the gently refluxing porphyrin solution. Immediately the color of the system changed from deep red to brown; after 10 minutes refluxing the system was opened to the air and let stand overnight. On standing, beautiful, macroscopic, octagonal platelets of metallic luster separated and were filtered off and washed repeatedly with 50 per cent acetic acid and then with water. If excess ferrous acetate had been added, no unchanged porphyrin was detectable. The yields in three such preparations were 88.1, 93.1, and 95.7 per cent of the theoretical.

In the case of iron coproporphyrin I complexes, solubility relationships modified the procedure. After the mixture was refluxed and had stood overnight, sufficient concentrated hydrochloric acid was added to react with the sodium acetate present. The system was then evaporated to dryness *in vacuo* and thoroughly extracted with 2 per cent hydrochloric acid, yielding a yellow filtrate of ferric chloride which exhibited no porphyrin or iron complex spectrum. The residue was a dark powdery material which was dried over phosphorus pentoxide. It was obviously impure as shown by the amounts of standardized, reduced phthiocol solution required in the titrations; it was estimated that the preparation contained about 70 per cent of iron coproporphyrin I complex. A colored impurity rendered spectrophotometric work impossible, but mathematical analysis of the titration data by the method of Reed and Berkson (17, 18) clearly demonstrated that the *main* product was iron coproporphyrin I chloride.

With this material reproducible potentiometric results were ob-

tained.   To establish the identity of the coporporphyrin I, the tetramethyl ester was prepared and characterized as already stated.   The reduced pyridine iron coproporphyrin I system in alkaline buffer solution showed the characteristic two-banded visible absorption with bands at 505 to 515 mμ and 540 to 550 mμ.

Description of Results

Descriptions of results will be abbreviated by referring to the propositions numbered in Paper I (2).

Potentiometric Measurements

The technique has been described in Papers I to III (2–4).

The observation of previous workers in this laboratory has been that the electrode potential is not steady in a solution of partially reduced metalloporphyrin if coordinating base be absent.   In certain instances a sufficient reason is found in the progressive precipitation of one of the components (see Taylor (3)).   On the other hand Davies (4) observed the same sort of drifts in systems the components of which were kept in solution by the addition of alcohol.   Here the known difficulties with alcohol solutions might have contributed.   With the water-soluble iron copropor-phyrins we find drifts of potential of about the same rate and duration.   The addition of a mediator did not improve matters.

Thus it is becoming evident that there is a distinct difference of behavior between metalloporphyrins and base metallopor-phyrins.   Apparently this is deep seated and will call for a special investigation.

Titrations—Tables I to III record typical titration data and are sufficiently representative to make unnecessary the recording of detail in all cases.

Symmetry of the titration curves indicated that $p = 1$ by Proposition I and rectification by Proposition XVI indicated that in each case $n = 1$.   Hence the process is $OB_q + e \rightleftharpoons RB_r$.

The low solubility of the components of the iron etioporphyrin system necessitated the use of ethanol (75 per cent) and water mixtures.   In such solutions progressive changes took place as was evident by the fading color.   Rapid titrations yielded the data of Table IV which have every appearance of significance.

Relation of E_b to pH—The procedure with the "glass electrode"

has been described (2). Since the behavior of a glass membrane in buffer solutions having high concentrations of nitrogenous bases and alcohol is of especial importance to this investigation, various tests were made to insure the reliability of the measurements. It was found that the potentials with a glass membrane

TABLE I

Titration of Pyridine Ferricoproporphyrin I with Reduced Phthiocol at Constant pH and Constant Pyridine Concentration

Demonstration that $n = 1$. Pyridine $= 1.05$ M; total pigment $\doteqdot 4.23 \times 10^{-5}$ M; phthiocol $\doteqdot 3.19 \times 10^{-4}$ M; pH of borate buffer $+$ pyridine $= 9.99$; $\mu = 0.1$; temperature $= 30°$; 100 per cent reduction at $y = 3.772$ ml.

y	$y - d$*	Reduction	$0.0601 \times \log \frac{S_r}{S_o}$	E_h observed	E'_b calculated	Deviation from average
ml.	*ml.*	*per cent*		*volt*	*volt*	*volt*
0.80	0.28	8.59	−0.0617	−0.0013	(−0.0630)	−0.0058
1.01	0.49	15.03	−0.0452	−0.0132	(−0.0584)	−0.0012
1.22	0.70	21.47	−0.0338	−0.0230	−0.0568	+0.0004
1.42	0.90	27.61	−0.0251	−0.0313	−0.0564	+0.0008
1.64	1.12	34.36	−0.0169	−0.0404	−0.0573	−0.0001
1.88	1.36	41.73	−0.0087	−0.0487	−0.0574	−0.0002
2.12	1.60	49.09	−0.0009	−0.0567	−0.0576	−0.0004
2.36	1.84	56.45	0.0067	−0.0644	−0.0577	−0.0005
2.66	2.14	65.66	0.0169	−0.0746	−0.0577	−0.0005
3.02	2.50	76.71	0.0311	−0.0873	−0.0562	+0.0010
3.24	2.72	83.46	0.0422	−0.0961	(−0.0539)	+0.0033
3.50	2.98	91.43	0.0618	−0.1083	(−0.0465)	+0.0107
Average..					−0.0572	

*Figures in the third decimal place of $y - d$ have not been recorded, since they are not physically significant, but, having appeared in the characteristics of the rectified curves, they were used automatically in the calculation of percentages of reduction.

were in orderly relation to the amounts of alkali added and were straight line functions of the alcohol concentration (to 80 per cent) in borate buffers with and without pyridine. In aqueous borate buffers the potentials were nearly straight line functions of the concentration of pyridine. It has been assumed *for purposes of comparison only* that the reproducible and orderly potentials

may be considered functions of the ratio of hydrogen ion activities on either side of the membrane as ordinarily formulated.

Fig. 1 shows that the pyridine iron etioporphyrin I system exhibits the relation $-dE_h/d$ pH $= 0.0601$ at 30°. This set of data is a decisive reinforcement of the argument that the carboxyl groups of the common metalloporphyrins have nothing to do with the "pH effect" within the range of pH shown. Etioporphyrin

TABLE II

Titration of Nicotine Ferricoproporphyrin I with Reduced Phthiocol at Constant pH and Constant Nicotine Concentration

Demonstration that $n = 1$. Nicotine $= 0.22$ M; total pigment $\approx 5.6 \times 10^{-5}$ M; phthiocol $\approx 5.31 \times 10^{-4}$ M; pH of phosphate buffer $+$ nicotine $= 11.25$; $\mu = 0.1$; temperature $= 30°$; 100 per cent reduction at $y = 3.402$ ml.

y	$y - d$	Reduction	$0.0601 \times \log \frac{S_r}{S_o}$	E_h observed	E'_b calculated	Deviation from average
ml.	*ml.*	*per cent*		*volt*	*volt*	*volt*
0.21	0.45	12.31	-0.0512	-0.0880	(-0.1392)	-0.0017
0.46	0.70	19.18	-0.0375	-0.1000	-0.1375	0.0000
0.61	0.85	23.30	-0.0311	-0.1063	-0.1374	$+0.0001$
0.83	1.07	29.34	-0.0229	-0.1141	-0.1370	$+0.0005$
1.10	1.34	36.76	-0.0141	-0.1225	-0.1366	$+0.0009$
1.40	1.64	45.00	-0.0052	-0.1322	-0.1374	$+0.0001$
1.70	1.94	53.25	0.0034	-0.1408	-0.1374	$+0.0001$
2.00	2.24	61.49	0.0122	-0.1498	-0.1376	-0.0001
2.30	2.54	69.73	0.0218	-0.1601	-0.1383	-0.0008
2.61	2.85	78.25	0.0335	-0.1717	-0.1382	-0.0007
2.80	3.04	83.46	0.0422	-0.1814	(-0.1392)	-0.0017
3.00	3.24	88.96	0.0545	-0.1940	(-0.1395)	-0.0020
3.20	3.44	94.45	0.0740	-0.2130	(-0.1390)	-0.0015
Average..					-0.1375	

See foot-note to Table I.

has no such groups. By inference a difference of 1 hydrogen or hydroxyl ion must be assigned to the coordination center of the reduced and oxidized forms of the metalloporphyrin. It is an arbitrary but reasonable assumption to ascribe a hydroxyl group to the coordination center of the oxidant. The symbol OOH will represent this assumption.

Fig. 1 presents the assembly of values of E_b obtained by in-

dividual titrations in the other aqueous solutions and shows the relation of potential to pH.   The lack of strict conformity to the calculated curves may be attributed partly to experimental errors in the several measurements needed to place each experimental point, partly to the instabilities that are revealed by the sensitive potentiometric method and that involve some personal judgment of what an equilibrium potential is (potential drifts *less than*

TABLE III

Titration of Cyanide Ferricoproporphyrin I with Reduced Phthiocol at Constant pH and Constant Cyanide Concentration

Demonstration that $n = 1$. Cyanide = 0.019 M; total pigment $\doteqdot$ 5.6×10^{-5} M; phthiocol $\doteqdot 4.25 \times 10^{-4}$ M; pH of borate buffer + sodium cyanide = 10.20; $\mu = 0.1$; temperature = 30°; 100 per cent reduction at $y = 3.075$ ml.; $d = 0$.

y	Reduction	$0.0601 \times \log \frac{S_r}{S_o}$	E_h observed	E'_b calculated	Deviation from average
ml.	*per cent*		*volt*	*volt*	*volt*
0.23	7.47	−0.0657	−0.1890	(−0.2547)	−0.0072
0.41	13.33	−0.0488	−0.2008	(−0.2496)	−0.0021
0.63	20.48	−0.0354	−0.2130	−0.2484	−0.0009
0.91	29.59	−0.0226	−0.2252	−0.2478	−0.0003
1.15	37.39	−0.0134	−0.2342	−0.2476	−0.0001
1.43	46.50	−0.0036	−0.2437	−0.2473	+0.0002
1.72	55.93	0.0062	−0.2535	−0.2473	+0.0002
2.05	66.66	0.0181	−0.2654	−0.2473	+0.0002
2.34	76.09	0.0302	−0.2773	−0.2471	+0.0004
2.63	85.52	0.0463	−0.2918	(−0.2455)	+0.0020
2.91	94.63	0.0749	−0.3092	(−0.2343)	+0.0132
Average..			−0.2475		

0.0001 volt per minute), and partly to inadequacy of corrections in converting the data to a common basis.   Such conversion involves not alone changing activity coefficients but also certain effects of larger order that will appear in the formulations given under "Discussion."   Nevertheless, the principal feature seems to be adequately demonstrated.   It is that systems containing nicotine and pyridine not only display the relation $-\Delta E_h/\Delta \, \mathrm{pH} = 0.0601$ in the more alkaline solutions, as Barron (1) and Davies

(4) found for similar systems containing other iron porphyrins, but they also display the transition to the "0 slope" which is a new feature.

By Propositions X and XI we may conclude that a species of oxidant associates with hydroxyl ion in the more alkaline solutions and not in the more acid solutions.   It does not follow that the

TABLE IV

Titration of Pyridine Ferrictioporphyrin I with Reduced Phthiocol in 75 Per Cent Ethyl Alcohol-Borate Buffer Mixture at Constant pH and at Constant Pyridine Concentration

Demonstration that $n = 1$. Pyridine $= 1.09$ M; total pigment $\doteqdot$ 4.76×10^{-5} M; phthiocol $\doteqdot 1.59 \times 10^{-4}$ M; pH of borate buffer + ethyl alcohol + pyridine $= 12.29$; temperature $= 30°$; 100 per cent reduction at $y = 6.483$ ml.

y	$y - d$	Reduction	$0.0601 \times \log \frac{S_r}{S_o}$	E_h observed	E'_b calculated	Deviation from average
ml.	ml.	per cent		volt	volt	volt
0.22	0.41	6.14	−0.0712	−0.1150	(−0.1862)	+0.0014
0.43	0.62	9.29	−0.0594	−0.1270	(−0.1864)	+0.0012
0.74	0.93	13.93	−0.0475	−0.1400	−0.1875	+0.0001
1.02	1.21	18.13	−0.0393	−0.1480	−0.1873	+0.0003
1.25	1.44	21.57	−0.0337	−0.1546	−0.1883	−0.0007
1.64	1.83	27.42	−0.0254	−0.1624	−0.1878	−0.0002
2.00	2.19	32.81	−0.0187	−0.1693	−0.1880	−0.0004
2.58	2.77	41.51	−0.0089	−0.1783	−0.1872	+0.0004
3.33	3.52	52.74	0.0028	−0.1903	−0.1875	+0.0001
3.85	4.04	60.54	0.0113	−0.1987	−0.1874	+0.0002
4.79	4.98	74.62	0.0281	−0.2159	−0.1878	−0.0002
5.28	5.47	81.97	0.0395	−0.2284	(−0.1889)	−0.0013
5.76	5.95	89.16	0.0550	−0.2424	−0.1874	+0.0002
Average..					−0.1876	

See foot-note to Table I.

apparent pK_a values are necessarily those of the ionization alone. This will be discussed later.

The cyanide system is invariant with pH like the similar systems in which cyanide is the coordinating substance.

Association Curves—Association curves for the iron coproporphyrin system and pyridine are typified by the case shown in Fig. 2.   By Proposition III, $K_o > K_R$.   The change of potential
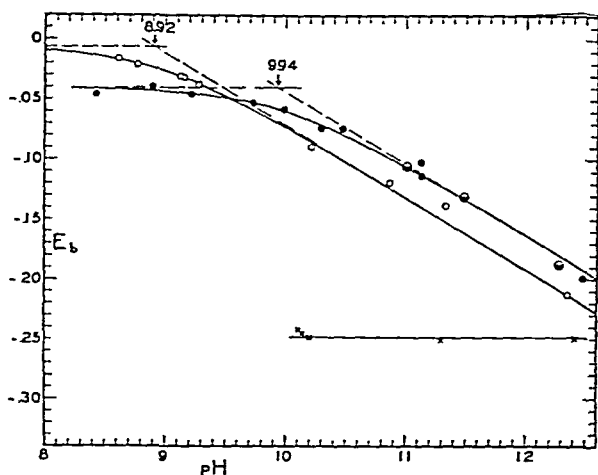
FIG. 1. Relation of E_b to pH as determined by individual titrations. $\times$ represents cyanide iron coproporphyrin; $S_o = S_r \doteqdot 2.5 \times 10^{-5}$ M; cyanide $\doteqdot 0.02$ M; $t = 30°$; $\mu \doteqdot 0.1$; reducing agent, reduced phthiocol. $\ominus$ represents pyridine iron etioporphyrin in water-ethanol (75 per cent), borate, and phosphate buffer solutions; $S_o = S_r \doteqdot 2.35 \times 10^{-5}$ M; pyridine $\doteqdot 1$ M; $t = 30°$; reducing agent, reduced phthiocol. $\bigcirc$ represents nicotine iron coproporphyrin; $S_o = S_r \doteqdot 2.5 \times 10^{-5}$ M; nicotine $\doteqdot 0.2$ M; $t = 30°$; $\mu \doteqdot 0.1$; reducing agent, reduced phthiocol. $\bullet$ represents pyridine iron coproporphyrin; $S_o = S_r \doteqdot 2.5 \times 10^{-5}$ M (except at point pH $= 11.16$ where $S = 1 \times 10^{-4}$ M); pyridine $\doteqdot 1$ M; $\mu \doteqdot 0.1$; $t = 30°$; reducing agent, reduced phthiocol.
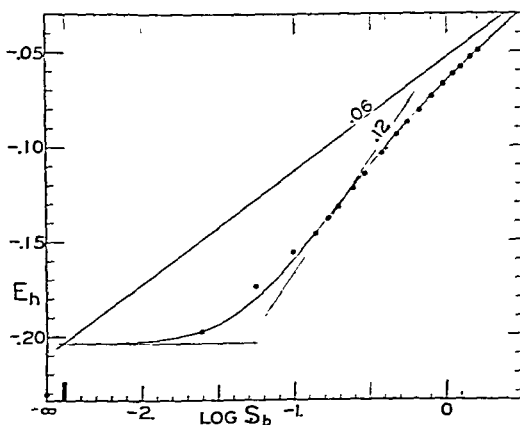
FIG. 2. Pyridine iron coproporphyrin. Relation of E_h to log S_b. The data are corrected to pH 10.02 (phosphate buffer) and 50 per cent reduction. $S = 5 \times 10^{-5}$ M; $t = 30°$. The empirical curve is drawn with $q = 1, r = 2$, $K_O = 0.6$, $K_R = 1.8 \times 10^{-3}$, $E'_0 = -0.2039$ volt (initial point neglected).

is so great as to signify that the ratio of K_O to K_R is large enough
for the application of Proposition VI and the maximum slope then
indicates that $r = 2$.   Application of Propositions VII and VIII
show that $q > 0$.   The data are not extensive enough to make
the approximate methods of analysis applicable with any certainty.
Therefore empirical curve fitting was resorted to.   The type curve
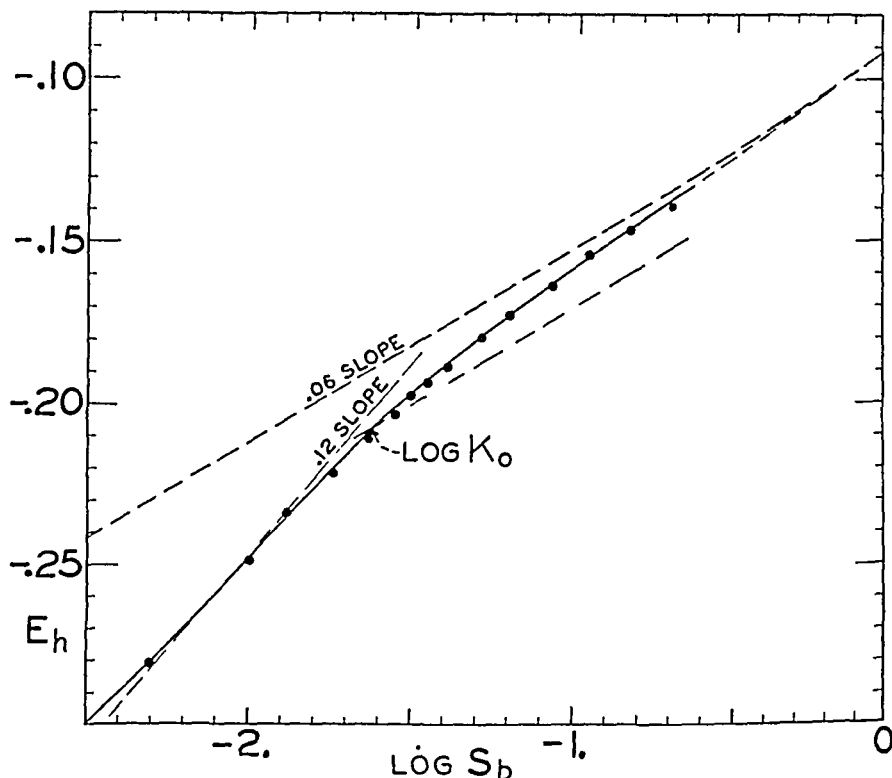
FIG. 3. Nicotine iron coproporphyrin.   Relation of E_h to log S_b. S
$= 5 \times 10^{-5}$ M.   The data are corrected to 50 per cent reduction and constant
pH.   pH $= 11.14$ (phosphate buffer); $\mu = 0.1$.   The curve is drawn with
$q = 1$, $r = 2$, $K_O = 2.25 \times 10^{-2}$, $K_R = 3 \times 10^{-6}$, $E'_0 = -0.3245$ volt.

drawn with the constants indicated in the legend of Fig. 2 are to
aid the eye in alignment of the experimental data, no reliance be-
ing placed on the estimate of K_O and K_R. K_R may be less than the
value used for the empirical curve if the initial point be significant.
The curve has been drawn with the assumption that $q = 1$, since
there is no evidence that $q = 2$; but empirical curve fitting will
succeed as well with the assumption that there is a stepwise associ-

ation and that the data extend only so far as to indicate the first step.

Empirical curve fitting had to be resorted to in dealing with the data of Fig. 3. Here it is much more certain that $r = 2$.

The case of cyanide association presented experimental difficulties. In several experiments the consequences appear in the poor alignment of the points. An attempt was made to stabilize the potentials by introducing indigotetrasulfonate as mediator. No distinct improvement followed. Fig. 4 represents one of the better series of measurements. Of particular interest is the decline of potential with increase in the concentration of cyanide
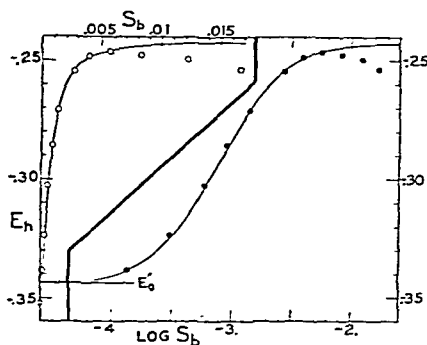
FIG. 4. Cyanide iron coproporphyrin. $S = 5 \times 10^{-5}$ M; $t = 30°$; per cent reduction $\doteqdot 50$. O represents the relation of E_h to S_b, ● relation of E_h to log S_b. The theoretical curves are drawn with $q = r = 2$, $K_O = 4 \times 10^{-6}$, $K_R = 8 \times 10^{-3}$, $E'_0 = -0.344$ volt (initial point neglected).

beyond about 0.005 M. Whether this be due to experimental error or to further addition of cyanide to the oxidant is uncertain. The over-all potential change is small, indicating a comparatively small ratio K_O/K_R. For this reason and also because of the uncertainties of points, graphical analysis is found to be unsatisfactory. Nevertheless, the theoretical curve given in Fig. 4 suggests that the main course of association follows that for the case of $q = r = 2$.

Comparison of Figs. 2, 3, and 4 shows that the value of K_R decreases in the order pyridine, nicotine, cyanide.

Spectrophotometric Measurements—The visible absorption bands of the oxidized metalloporphyrins change so little with addition

of pyridine or nicotine that the available spectrophotometer was inadequate for photometric measurements of the precision required for estimation of constants.

<center>DISCUSSION</center>

The data here presented confirm in principle several of the relations discussed by Taylor (3) and by Davies (4) and in particular they support Davies' theory of the decisive effect of competition between the nitrogenous bases and hydroxyl ion for bonds of the coordination shell.

We now meet a new feature that needs careful consideration. What is the significance of the distinctive bends of the E_h-pH curves of Fig. 1?

Since Davies' systems provided no evidence to the contrary, he postulated but one acid dissociation constant. Is it possible to account for the distinctive values of the apparent pK_a by use of his Equations 3 and 4 (4)? In the end a decision must rest upon the establishment of consistency among the numerical values of constants that are not yet available; but to provide a basis of discussion we may take a broad view by postulating the several species found in the following equations of equilibrium.

$$\frac{[R][B]^2}{[B_2R]} = K_R \quad (1) \qquad \frac{[BO][B]}{[B_2O]} = K_{O_1} \quad (2) \qquad \frac{[O][B]}{[BO]} = K_{O_2} \quad (3)$$

$$\frac{[O\overline{O}H][H^+]}{[O]} = K_{h_1} \quad (4) \qquad \frac{[B_2O\overline{O}H][H^+]}{[B_2O]} = K_{h_2} \quad (5) \qquad \frac{[BO\overline{O}H][H^+]}{[BO]} = K_{h_3} \quad (6)$$
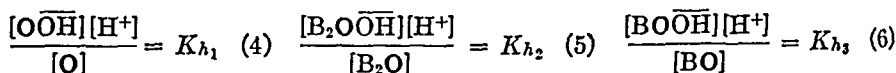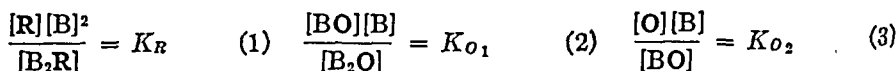
With these equations, and with obvious definitions of S_o and S_r, one may derive

$$E_h = E_0 + 0.06 \log \frac{S_o}{S_r} + 0.06 \log \frac{K_{O_1}K_{O_2}}{K_R} +$$

$$0.06 \log \frac{[H^+](K_R + [B]^2)}{K_{O_1}K_{O_2}([H^+] + K_{h_1}) + K_{O_1}[B]([H^+] + K_{h_3}) + [B]^2([H^+] + K_{h_2})} \quad (7)$$

When $[H^+]$ is much greater than K_{h_1} and K_{h_2} and K_{h_3}, and for any constant value of $[B]$, the slope will be $-dE_h/d$ pH $= 0$.

When $[H^+]$ is much less than K_{h_1} and K_{h_2} and K_{h_3}, and for any constant value of $[B]$, the slope will be $-dE_h/d$ pH $= 0.06$.

For each of these sets of conditions there will be a family of

straight lines.   Those of any pair, representing a case of constant [B], will intersect where

$$[H^+] = \frac{K_{o_1}K_{o_2}K_{h_1} + K_{o_1}K_{h_1}[B] + K_{h_2}[B]^2}{K_{o_1}K_{o_2} + K_{o_1}[B] + [B]^2} \qquad (8)$$

If [B] = 0, Equation 8 reduces to $[H^+] = K_{h_1}$; in other words the intersection of projections of the two branches of the E_h-pH curve is where pH = pK_{h_1}.

Should the terms in $[B]^2$ predominate in the sums of Equation 8, the intersection would be where pH = pK_{h_2}. Should the terms in [B] predominate in the sums of Equation 8, the intersection would be where pH = pK_{h_3}.

In the cases at hand the evidence is that the terms in $[B]^2$ have values of the second order and that [B] is somewhat greater than K_{o_2}. Therefore it would appear that the intersection is given approximately by $[H^+] = (K_{o_2}K_{h_1}/[B]) + K_{h_3}$ or in other words that it is considerably different from pH = pK_{h_1} and displaced a little from pH = pK_{h_3}.

Thus it would appear that the values of pK_c revealed in Fig. 1 are distinctive, largely, but not wholly, because they are determined by the nature of the associating base that enters together with hydroxyl ion to form the species $BO\overline{OH}$.

The case of the cyanide system can be reconsidered with Equation 7.   Here the terms in $[B]^2$ predominate at high concentrations of cyanide.   To obtain dE_h/d pH = 0, K_{h_2} must be much less than $[H^+]$.   In other words the introduction of the negatively charged cyanide ions repels $\overline{OH}$ even at high concentrations of the latter.

Having thus mapped several of the gross features of these systems, we can now see several important relations that should be put to experimental test.   It was the fate of this exploration that when the outline map had been completed the stock of coproporphyrin was exhausted.

SUMMARY

1. Some details of the synthesis of coproporphyrin I are mentioned.

2. A preliminary, spectroscopic study of the reaction

$$Fe^{++} + porphyrin \rightleftharpoons ferroporphyrin + 2H^+$$
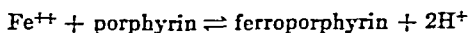
in glacial acetic acid indicated that the "left to right" reaction is favored by low proton activity. Accordingly, the strong base sodium acetate was used in the preparation of ferricoproporphyrin I and ferrictioporphyrin I.

3. The oxidation-reduction potentials during titration of the pyridine, nicotine, and cyanide complexes of synthetic iron coproporphyrin I have been measured in alkaline, buffered, aqueous solutions and of pyridine iron etioporphyrin I have been measured in alkaline, buffered, ethanol (75 per cent) solutions. In every case the evidence indicates that all species are monomers and that 1 equivalent per mole is involved in the oxidation-reduction.

4. At constant, high concentration of pyridine the pyridine iron etioporphyrin system in aqueous ethanol exhibits the relation $-\Delta E_b/\Delta \text{ pH} = 0.06$. Since there are no carboxyl groups in this system, the result supports the assumption that a hydroxyl ion coordinates in the metal ion coordination shell.

5. At constant, high concentration of coordinating base the pyridine and nicotine iron coproporphyrin systems give $-\Delta E_b/\Delta \text{ pH} = 0.06$ at high pH. This value changes to 0 at low pH with apparent pK_a values of 9.9 for the pyridine system and 8.9 for the nicotine system.

6. At constant, high concentration of cyanide the cyanide iron coproporphyrin system gives $\Delta E_b/\Delta \text{ pH} = 0$.

7. The evidence is good that 2 molecules of cyanide, or pyridine or nicotine, associate with 1 of ferrocoproporphyrin. Orders of magnitude of the values of K_R are given. The values of K_R increase in the order cyanide, nicotine, pyridine.

8. It is certain that these bases coordinate with ferricoproporphyrin but the number (q) is uncertain in the cases of pyridine and nicotine although undoubtedly it is 2 in the case of cyanide.

9. To account for the distinctive, apparent dissociation constants of pyridine and nicotine ferricoproporphyrin it is shown that it is necessary to increase the number of components of Davies' model system.

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METALLOPORPHYRINS

V. A SPECTROPHOTOMETRIC STUDY OF PYRIDINE COPROPORPHYRIN I

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Previous, potentiometric studies (1–3) in this laboratory of base metalloporphyrins were not supplemented adequately by spectrophotometric measurements either because the differences between the extinction coefficients of the components of such systems were not great enough or because the available spectrophotometer in our hands lacked the required precision. In an effort to meet the instrumental requirement at small expense the device described below was constructed with instruments at hand.

In addition to the use of this photoelectric spectrophotometer for the purposes described in the "Supplementary note" to Taylor's paper (1) it has now been applied in an effort to clarify certain questions left undecided by Vestling's (3) potentiometric exploration of systems containing iron coproporphyrin I.

Photoelectric Spectrophotometer

Since this instrument was an improvisation adapted to the use of an available, but expensive, White, double potentiometer (4) and will doubtless not be copied, its description need not be given with more detail than is necessary to the understanding of the principles employed.

The light source was a 50 candle power, automobile headlight lamp supplied with current as follows: From the 110 volt D.C. main, current was sent through a bank of ballast lamps in parallel and at a potential drop of 50 volts. The current, partly stabilized by these ballast lamps, was further stabilized by floating two large 6 volt storage-batteries in parallel across the lamp

terminals.   Shunt resistances were adjusted until the batteries were charging very slightly.   All connections except at the sliding contacts of resistors were soldered.   Occasionally the residual fluctuations of current were so disturbing as to necessitate abandonment of measurements.   Usually the illumination was remarkably steady.

A large, Bausch and Lomb, wave-length spectrometer served as monochromator.   Light of the selected wave band was sent through solvent and solution alternately according to the substitution method.   In each case the light fell upon a single,
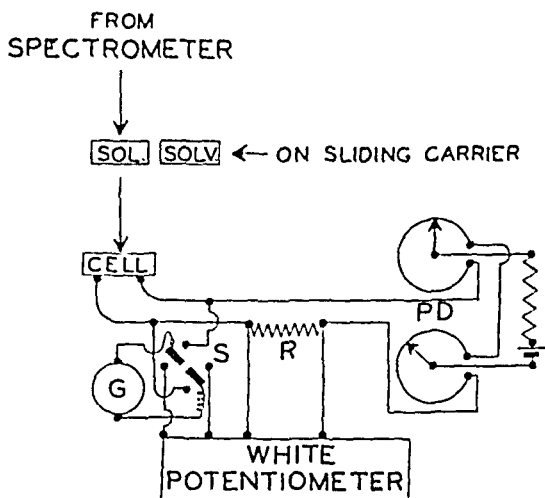
FIG. 1. Elementary diagram of parts of the photoelectric spectrophotometer.   *Cell* General Electric barrier layer, selenium cell, *G* galvanometer, *S* switch, *R* fixed resistance, *PD* potential divider.

General Electric, selenium, barrier layer cell the output of which was measured as follows:

A prerequisite said to be essential to a linear relation between current output and energy received at constant wave-length is zero resistance across the cell's terminals.   To meet this, current from the potential divider *PD* (Fig. 1) was regulated until the galvanometer *G* showed no potential difference across the cell's terminals.   Then the galvanometer was switched by means of *S* to its use with the White potentiometer and the current flowing through the fixed resistance *R* was measured potentiometrically. Fig. 1 shows but one potential divider.   Two were used, one for adjustment when solution was in train, the other for adjustment

when solvent was in train. Switches, not shown, also provided that one part of the double potentiometer could be used when solu-

TABLE I

Test of Photoelectric Spectrophotometer with Glasses

Slit 0.05 mm.; eyepiece shutter admitting band about 5 mμ wide at mμ 600; Cell A, General Electric selenium barrier layer cell; Cell B, Westinghouse cell.

Cell	Glass	mμ	Transmittance		Deviation T
			Observed	Bureau of Standards	
A	Blue	435.8	0.803	0.811	−0.008
		491.6	0.518	0.528	−0.010
		546.1	0.147	0.154	−0.007
		578.0	0.137	0.136	+0.001
		587.6	0.0747	0.0740	+0.0007
		610.0	0.0720	0.0750	−0.0030
		640.0	0.0612	0.0654	−0.0042
		667.8	0.131	0.146	−0.015
		720.0	0.842	0.82	+0.022
A	Amber	460.0	0.0333	0.0270	+0.0063
		491.6	0.0968	0.1025	−0.0057
		520.0	0.2330	0.240	−0.007
		546.1	0.396	0.400	−0.004
		578.0	0.569	0.583	−0.014
		620.0	0.682	0.685	−0.003
		670.0	0.704	0.707	−0.003
		720.0	0.704	0.695	+0.009
A	Didymium	501.6	0.781	0.781	0.000
		530.0	0.386	0.393	−0.007
		546.1	0.848	0.864	−0.016
		578.0	0.0582	0.0668	−0.0086
		587.6	0.0250	0.0280	−0.0030
		650.0	0.895	0.898	−0.003
		700.0	0.875	0.880	−0.005
B	Didymium	501.6	0.782	0.781	+0.001
		530.0	0.393	0.393	0.000
		546.1	0.855	0.864	−0.009
		578.0	0.0638	0.0668	−0.003
		587.6	0.0268	0.0280	−0.0012

tion was in train and the other when solvent was in train. Thus, after preliminary settings of potential dividers and potentiometers, solvent and solution could be interchanged rapidly by the

sliding carriage and final readings taken rapidly. Rapidity of readings is essential to the proper use of the substitution method, since some fluctuation of the light is inevitable. The chief drawback encountered is the small, residual drift, commonly called "fatigue," of a barrier layer cell after change of light intensity. A Westinghouse "Photox" cell having a very low "fatigue" characteristic was also used but without materially improving the performance of our instrument.

Table I summarizes measurements of three glass disks the transmittances of which were reported some years previously by the Bureau of Standards. At several of the wave-lengths the Bureau used a mercury or helium lamp to eliminate the error of finite slit width, while we used slit widths of the order of 0.05 mm. and an eyepiece shutter that admitted a band which at 600 mμ was about 5 mμ wide. An equally serious source of discrepancy lies in the fact that while the readings on the wave-length drum were calibrated by use of a helium Plücker tube there remain slight but important errors in the setting of the eyepiece shutter. Since several of the points selected by the Bureau are on very steep parts of the transmittance curves of these glasses, the combined uncertainties mentioned are sufficient to account for the discrepancies in Table I. The mean error of all measurements is ± 0.0016 on the assumption that the Bureau's values are absolute. The Bureau claims an uncertainty of ± 1 per cent of each value.

For the purposes at hand it is less important that points on an absorption curve be accurately placed than that $-\log T$ be strictly proportional to the concentration of absorbing component when wave-length drum, slit, and shutter are in fixed positions. Accordingly, a test of conformity to Beer's law was made with copper sulfate in 2 M ammonia solutions as recommended by Drabkin and Austin (5). For such solutions they attained a precision with a Bausch and Lomb spectrophotometer much better than we have attained with this type of instrument; but, as Table II illustrates, our results with the photoelectric device are more consistent over a wider range of dilution.

The absorption tubes used in this investigation were 1 cm. tubes of the highest precision made by the American Instrument Company.

TABLE II

Test of Beer's Law

Volumetric dilutions of 0.1 M CuSO$_4$ in 2 M NH$_3$; cell length 1 cm.; slit 0.1 mm.; shutter admitting band 5 mμ wide at 600 mμ; General Electric selenium barrier layer cell; $-\log T = \epsilon cl$; M = mean error of all observations $= \pm \sqrt{\Sigma(v^2)/(n(n-1))}$.

Values of ε, Series I

CuSO$_4$, M	0.05	0.05	0.0308	0.01	0.005	0.00308	0.002	0.0005	0.0002	Average	M
620 mμ	(55.31)*	56.22†	58.75	58.98	58.76	58.85	58.80	59.00	(64.00)	58.91	0.06

Values of ε, Series II

CuSO$_4$, M	0.04	0.02	0.01	0.005	0.0025	0.00125	0.000625	Average	M
650 mμ	(55.09)	57.11	57.09	56.72	56.44	57.76		57.02	0.22
630 "	57.24	59.07	58.92	59.02	60.24	59.28		58.96	0.34
600 "	55.14	57.23	56.91	56.40	56.16	56.00	55.08	56.22	0.27
570 "	47.08	48.19	48.00	48.12	48.52	48.32	49.76	48.51	0.26
550 "	38.00	39.07	38.82	39.02	41.08	40.00	(45.28)	39.48	0.36

Values of ε, Drabkin and Austin

CuSO$_4$, M	0.05	0.025	0.0125	0.01	0.00312	Average	M
650 mμ	53.2	51.8	53.6	54.8	56.2	53.92	0.75
630 "	57.0	54.0	57.0	60.2	58.8	57.52	0.05
600 "	57.0	55.4	57.0	59.2		57.15	0.55
570 "	48.2	48.2	50.8	50.6	50.0	49.56	0.57
550 "	38.6	38.8	39.8	39.0	30.4	38.52	0.57

The values in parentheses are not included in the averages.

* There was so little transmittance in a 1 cm. layer that readings were made by galvanometer deflection.

† Used vessel 0.5 cm. deep.

Analyses of Data

The objective methods outlined by Clark, Taylor, Davies, and Vestling (6) for the analysis of spectrophotometric data were used in all cases. In particular, constants were estimated from the properties of the rectified curves. For brevity the propositions they proposed for test will be referred to here by number.

Results

Fig. 2 summarizes the data obtained with ferricoproporphyrin alone (absence of coordinating base) in buffer solutions of different pH values. The data were analyzed by the rectification method of Proposition XIV and for the assumption that 1 proton associates with or neutralizes the effect of 1 hydroxyl ion per iron atom. The value of pK_a and values of ϵ'_1 and ϵ'_2 calculated from the properties of the rectified curve were used in constructing the type curve of Fig. 2 wherein there is seen a good agreement with the experimental points. The inset of Fig. 2 indicates the small range of $-\log T$ (ϵ') within which the changes at mμ 600 had to be measured with a precision sufficient for the purposes.

Fig. 3 shows that at high, constant concentration of pyridine the absorbing species vary with pH in accordance with the law applied in Fig. 2. The same objective methods of analysis were used.

Fig. 4 represents the change of degree of association between pyridine and ferricoproporphyrin as a function of log [pyridine] at two different values of pH. The same sort of objective analyses was employed again. The agreement with the theoretical form of curve for concerted addition of 2 molecules of pyridine per molecule of metalloporphyrin is both satisfactory and surprising. Exception may be taken in the case of the measurements at pH 12.7, since the two uppermost points do not conform. Therefore the basis for the placement of the theoretical curve is given in Fig. 5. This illustrates the application of Proposition XIV. It is important to note that the method used gives weight to all points falling on or near a straight line and is not dependent upon the measurements of the limiting transmittances T_1 and T_2.

Analyses of Vestling's (3) potentiometric data have shown no reason for believing that ferrocoproporphyrin and its base com-
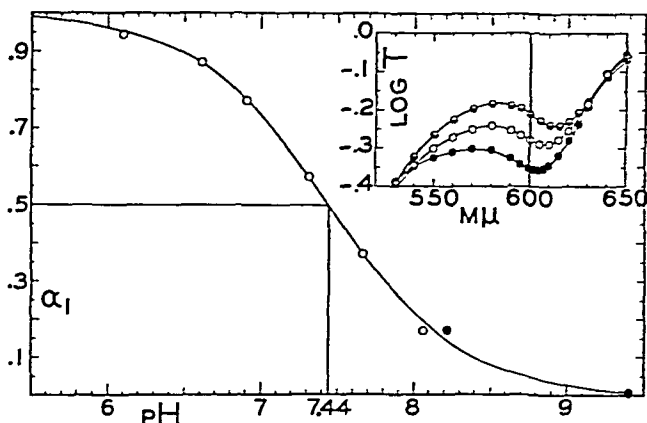
FIG. 2. Relation of α_1 to pH; ferricoproporphyrin; O represents phosphate buffers, ● borate buffers; measurements at mμ 600. The theoretical curve conforms to pH $= pK_{h_1} + \log (1 - \alpha_1)/\alpha_1$. See Equation 7 for the case of [B] $= 0$; $pK_{h_1} = 7.44$; $K_{h_1} = 3.6 \times 10^{-8}$. Inset, log transmittance curves of ferricoproporphyrin I in solutions of different pH values; $S_o = 9.4 \times 10^{-5}$ M (approximate); ◐ at pH 6.6, O at pH 7.9, ● at pH 9.6.
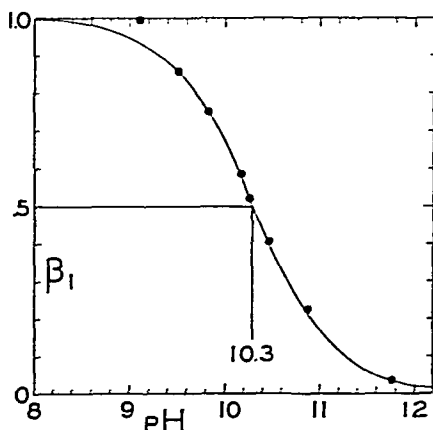
FIG. 3. Relation of β_1 to pH at high concentration of pyridine (2.48 M). The theoretical curve conforms to pH $= pK_{h_2} + \log (1 - \beta_1)/\beta_1$. See Equation 5 for the case in which [H$^+$] $<< K_{h_1}$ and [B]^2 $>> k_0$; $pK_{h_2} = 10.3$; $K_{h_2} = 5 \times 10^{-11}$.
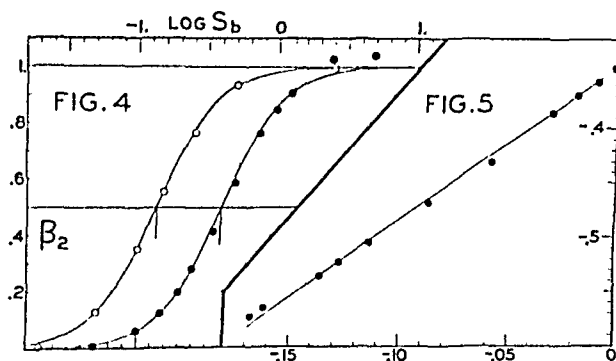
FIG. 4. Association curves; ferricoproporphyrin (9.44×10^{-5} M) + pyridine; ● at pH = 12.7. The theoretical curve conforms to 2 log [B] = log k_0 + log $\beta_2/(1 - \beta_2)$. Combine Equations 6 and 4 and assume K_{h_1} and K_{h_2} greater than [H⁺]. $k_0 = 0.155$. ○ at pH = 9.1; see discussion.

FIG. 5. Rectification of relation between observed log transmittance and 2 log [B] in the case of the measurements of association at pH 12.7. Ordinate, log T; abscissa, $10^p(\epsilon'_0 - \epsilon')/(1 - 10^p)$. See Proposition XIV (6). The lower points of Fig. 5 correspond to the upper points of Fig. 4, right-hand curve.
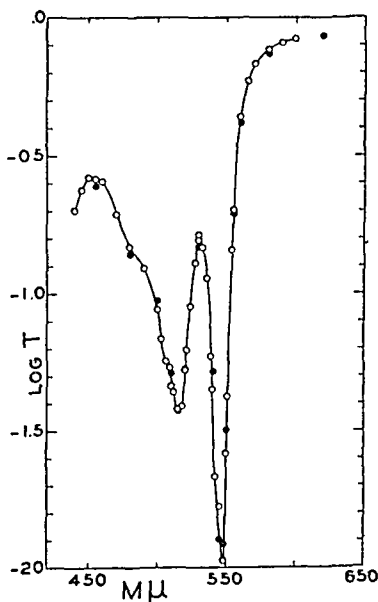
FIG. 6. Absorption curve of pyridine ferrocoproporphyrin I. ○ at pH 12.4; ● at pH 8.5; $S = 1.09 \times 10^{-4}$ M; $S_b = 2.48$ M; 1 cm. cell.

plexes change with pH. Fig. 6 supports this conclusion within the range of pH examined, since points on the spectrophotometric absorption curve of pyridine ferrocoproporphyrin in solution at

pH 12.4 are very closely duplicated by those measured at pH 8.5.

Measurements of the association of pyridine with ferrocoproporphyrin were made less certain than those of the oxidized system, because the solid dithionite ($Na_2S_2O_4$), added as a reducing agent, produced a slight turbidity. The initial measurements made at mμ 548, shown in Fig. 7, were puzzling. Not only
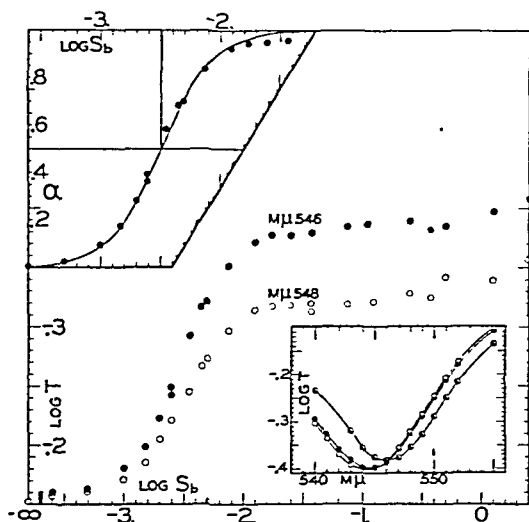
FIG. 7. Ferrocoproporphyrin + pyridine. Relation of observed log T to log S_b; ● at mμ 546, O at mμ 548. Right insert, shift of log transmittance minimum with increase of pyridine; O 0.037 M pyridine, ● 0.906 M pyridine, ◐ 2.48 M pyridine. Left insert, relation of α to log [B]. The theoretical curve conforms to 2 log [B] = log K_R + log $\alpha/(1 - \alpha)$ where $\alpha = [B_2R]/S_r$; $K_R = 1.02 \times 10^{-5}$.

did the application of Proposition XIV fail to yield a good rectification, the variability and continued shift of transmittance as the concentration of pyridine reached the higher levels were most disconcerting. Now it was noticed that with the larger increases of pyridine concentration there was a distinct shift of the "absorption peak," illustrated by the right-hand inset of Fig. 7. Unfortunately this experiment was made with the last available material and it was not proved that the intersection of the curves shown is a true isobestic point. Nevertheless, it was guessed

that it might be and it was conjectured that if measurements were taken at mμ 546 there might be obviated some of the interference with what appeared to be a new species forming at the higher concentrations of pyridine. Accordingly, the same solutions were remeasured at mμ 546. Then the method of rectification, when confined to the points on the sigmoidal part of the experimental curve, yielded constants with which the curve in the upper left-hand inset of Fig. 7 was constructed. The agreement is now satisfactory for all except the uppermost points and again the indication is that 2 molecules of pyridine coordinate, at least for the first stage.

DISCUSSION

The spectrophotometric data for pyridine ferrocoproporphyrin, within the limitation specified, confirm Vestling's (3) potentiometric results in the following respects. No acid ionization is involved between pH 8.5 and 12.4; $K_R < K_0$; $r = 2$. The photometric evaluation of K_R is probably more accurate than Vestling's, since he was uncertain of the reliability of potentials taken at the lower concentrations of pyridine for reasons that he has stated. Vestling's curve ((3) Fig. 2) is constructed with the assumption of a value of K_R very much higher than the photometric value but if greater weight be given to his measurements at lower pyridine concentrations an estimate that K_R is of the order of 1×10^{-5} can be obtained. This would be in agreement with the value here reported. The difficulty that remains is in accounting for the behavior of the system at high concentrations of pyridine. Explanation had best await more data. Nevertheless, it should be mentioned that if the spectral data have indicated the formation of a new pyridine ferrocoproporphyrin at very high concentrations of pyridine, the consequences would be most disturbing. The formation of such a new species of reductant would give an upward trend to the potentiometric association curve and the effect would overlap the effect of association with oxidant, making extremely difficult the estimation of the individual values of q and r.

To formulate the photometric data for the oxidized systems assume the hypothetical species found in the following equations of equilibria and attached deductions.

$$\frac{[O][B]^2}{[B_2O]} = K_o \qquad (1)$$

$$\frac{[O\overline{OH}][H^+]}{[O]} = K_{h_1} \qquad (2)$$

$$\frac{[B_2O\overline{OH}][H^+]}{[B_2O]} = K_{h_2} \qquad (3)$$

and the unnecessary but convenient relation

$$\frac{[O\overline{OH}]\,[B_2]}{[B_2O\overline{OH}]} = K_o \frac{K_{h_1}}{K_{h_2}} = k_o \qquad (4)$$

Note that K_o of Equation 1 is the $K_{o_1}K_{o_2}$ of Vestling's equations and that species with one associated base are left out of consideration in order to abbreviate the treatment for the purposes at hand.

With the obvious definition of S_o derive

$$\beta_1 = \frac{[B_2O]}{S_o} = \frac{[H^+][B]^2}{Y} \qquad (5)$$

$$\beta_2 = \frac{[B_2O\overline{OH}]}{S_o} = \frac{K_{h_2}[B]^2}{Y} \qquad (6)$$

$$\alpha_1 = \frac{[O]}{S_o} = \frac{K_o[H^+]}{Y} \qquad (7)$$

where

$$Y = K_o([H^+] + K_{h_1}) + [B]^2([H^+] + K_{h_2}) \qquad (8)$$

Fig. 2 is a confirmation of Equation 2 as regards form. Since no independent measurement, such as might flow from a potentiometric study at low pH, is available, we must not overlook the possibility that the spectrometer has picked up evidence of an impurity that behaves as an acid-base indicator. But, granting that the data are significant, they yield, so far as we know, the first quantitative measurement of the acid dissociation constant of a ferriporphyrin. Since $[B] = 0$, Equation 7 can be recast to the form $pH = pK_{h_1} + \log (1 - \alpha_1)/\alpha_1$ with which the curve of Fig. 2 has been plotted.

Since this experiment shows that $K_{h_1} = 3.6 \times 10^{-8}$, the high alkalinity of the association experiment at pH 12.7 (Fig. 4) re-

quires that the ferriporphyrin be almost exclusively in the form $O\overline{O}H$. The data can be formulated by Equation 4 but before commitment to this let us examine an important matter. Equation 4 specifies the existence of the curious species $B_2O\overline{O}H$, curious because it may imply that, in addition to four coordination bonds between the iron ion and the four pyrryl nitrogens, there are two holding the pyridines and one holding the hydroxyl ion. This assumption of seven bonds, presumably coordinative, violates the rule that the coordination number of iron is usually six. More than six would be improbable in a compound of this sort.[1] Therefore let us see what can be done with only Equations 1 and 2. With these assumptions the data of Fig. 4, case of pH 12.7, would have to be described by the product of Equations 1 and 2 rearranged to Equation 9.

$$\frac{[O\overline{O}H][B]^2}{[B_2O]} = \frac{K_O K_{h_1}}{[H^+]} = 0.155 \tag{9}$$

Whence, from a knowledge of the values of K_{h_1} and $[H^+]$, we should estimate K_O to be about 8.6×10^{-7}.

By a similar argument the experiment of Fig. 3 should be described by

$$\frac{[O\overline{O}H][H^+]}{[B_2O]} = \frac{K_O K_{h_1}}{[B]^2} = 5 \times 10^{-11} \tag{10}$$

Whence K_O should be about 8.5×10^{-3}. The discrepancy between these estimates involves a factor of about 10,000 and forces the rejection of the postulate on which the estimates were predicated. In other words we seem forced to the assumption of the very unorthodox species $B_2O\overline{O}H$.

A somewhat similar situation was indicated by Davies' curve for the association between iron protoporphyrin and pyridine. Treatment of those data, as found, required stepwise addition of pyridine to the dimeric ferriprotoporphyrin, the last addition forming a compound that was formulated as $B_4O_2(\overline{O}H)_2$. However, it was questioned whether corrections of the data to a common basis had been adequate to assure this conclusion and the matter was left in abeyance.

Independent evidence of acid dissociation constants different

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[1] Without evidence it is gratuitous to suggest that a seventh coordination bond may be a hydrogen bond not involving the iron.

from K_{h_1} was established by Vestling when he found that the E_b-pH curve of pyridine iron coproporphyrin undergoes an inflection near pH 10 and of nicotine iron coproporphyrin undergoes an inflection at slightly lower pH. Vestling was able to escape from the dilemma because his limited data permitted either the assumption that $q = 1$ or $q = 2$ and by adopting the first he could specify the species $BO\overline{OH}$ which does not violate the rule of coordination.

Apparently then the association experiment of Fig. 4, case of pH $= 12.7$, gives a first order approximation of k_0 and the experiment of Fig. 3 a first order approximation of K_{h_2}. To check the absence of serious interferences by other species Equation 3 is used to show that $[B_2O\overline{OH}]/[B_2O]$ is about 250 in the case to which Equation 4 applies so that there is very little interference by B_2O. Also Equation 4 is used to show that $[O\overline{OH}]/[B_2O\overline{OH}]$ is about 0.025 in the case to which Equation 3 applies so that there is little interference by the species $O\overline{OH}$.

However at pH 9.1 (see Fig. 4) $[B_2O\overline{OH}]/[B_2O] = 7.95$ by Equation 3. Therefore the three species $B_2O\overline{OH}$, B_2O, and $O\overline{OH}$ concur and this might seem to render dubious an interpretation based on the assumption of but two absorbing species. If the transmittance by the base metalloporphyrin were in reality the transmittance by the sum $[B_2O\overline{OH}] + [B_2O]$, we shall have to consider first whether or not the ratio of the concentrations of these two species varied considerably as pyridine was added. For this purpose consider Equation 3. Evidently the ratio could not have changed if $[H^+]$ were constant as we must assume it was. Therefore the transmittance by the sum $[B_2O\overline{OH}] + [B_2O]$ can be expressed by $-\log T_a = k' [B_2O\overline{OH}]$ where the constant k' is a function of the transmittance coefficients of $B_2O\overline{OH}$ and B_2O and of $[H^+]$ and K_{h_2}. In other words the *form* of the curve should not be affected in this case by the presence of three species instead of two, but the transmittance by the base metalloporphyrin should be interpreted as that of the sum $[B_2O\overline{OH}] + [B_2O]$.

Accordingly, the applicable equation is

$$\frac{[O\overline{OH}][B]_2}{[B_2O\overline{OH}] + [B_2O]} = \frac{K_0 K_{h_1}}{([H^+] + K_{h_2})} = \frac{k_0 K_{h_2}}{([H^+] + K_{h_2})} = 0.0158 \quad (11)$$

Whence $k_0 = 0.31$. The direct estimate of k_0 was 0.155 so that there remains a discrepancy involving a factor of 2. This may be greater than the capability of the method but it may not be greater than the uncertainty due to dependence on the single series of measurements for each case, to which we were limited by the available material.

There remains the obvious and disturbing conflict between the spectrophotometric evidence that $q = 2$ and Vestling's potentiometric evidence that $q = 1$. Attempts to find a reconciliation might run the usual course of reaching a volume of comment inversely proportional to the volume of significant data. Therefore we prefer to report now what the experiments seem to show and to await a new supply of coproporphyrin I.

SUMMARY

A photoelectric spectrophotometer has been improvised and two tests of its performance reported.

Photometric measurements of an ionization constant of ferricoproporphyrin I give a pK_{h_1} value of 7.44, and of an ionization constant pertaining to pyridine ferricoproporphyrin give a pK_{h_2} value of 10.3. At pH 12.7 the measurements indicate that 2 molecules of pyridine add concurrently and that the dissociation constant of the pyridine complex is 0.155. A third series of measurements with the limited material at pH 9.1 served as a check upon the calculations and these led to the conclusion that a consistent account of the relations could be given by postulating a species in which 2 molecules of pyridine and 1 of hydroxyl ion are coordinated with the ferriporphyrin.

No sign of an acid ionization between pH 8.5 and 12.4 was de-

tected in the case of ferrocoproporphyrin I. One experiment indicated that 2 molecules of pyridine add concurrently, at least in a first stage of association, and that the dissociation constant of this complex is about 1×10^{-5}. Whether further addition of pyridine occurs at higher concentrations is left open to question.

The results confirm those parts of Vestling's potentiometric study of which he was certain and extend the knowledge of the system.

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A PHOTOELECTRIC METHOD FOR THE MICRODETERMINATION OF POTASSIUM IN BLOOD PLASMA BY THE CHLOROPLATINATE PRECIPITATION

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In 1928 Shohl and Bennett (8) adapted the precipitation as the chloroplatinate to the determination of potassium in 1 cc. samples of serum. In 1938 Consolazio and Talbott (1) modified this method by using quartz centrifuge tubes in the ashing procedure, a change which eliminated the danger of loss in transferring the samples. Both groups of investigators mentioned the use of the ordinary colorimeter for reading the concentration of potassium iodoplatinate in the final solution, although both preferred titration of the potassium iodoplatinate solution with sodium thiosulfate.

It is the purpose of the present paper to present a further modification of the chloroplatinate precipitation method for use with 0.3 cc. of blood plasma, the final step in the procedure being based upon the photoelectric measurement of the reddish orange color of the potassium iodoplatinate in solution. The maximum absorption of this color has been found to be 490 mμ. Various concentrations of this substance when measured in the Evelyn photoelectric colorimeter[1] (3) with the green Filter 490 (transmission limits 465 to 530 mμ, maximum transmission 490 mμ) give a smooth curve by which differences in concentration of less than 1 per cent can be detected. In the normal and pathological range of potassium concentrations in human plasma this absorption curve has been found to follow Beer's law when the final dilution of a 0.3 cc. sample is made up to 25.0 cc.

[1] Made by the Rubicon Company, Philadelphia.

Method

Reagents—

1. 6 N H_2SO_4 containing 300 milliequivalents per liter of Na_2SO_4.
2. Concentrated HNO_3.
3. 20 per cent chloroplatinic acid in 1 N HCl.
4. Absolute alcohol saturated with K_2PtCl_6.
5. 2 N potassium iodide.

Procedure

Ashing—To 0.3 cc. of plasma in a 15 cc. conical quartz centrifuge tube[2] is added 0.3 cc. of 6 N H_2SO_4 which contains 300 milliequivalents per liter of Na_2SO_4. At the same time 0.1 cc. of concentrated HNO_3 is added and the tube is placed in a boiling water bath for 30 to 60 minutes. The tube is then placed in a sand bath at 120° (the temperature is taken at the tube tip by placing a thermometer in the sand to the same depth as the tube) until only a dark brown drop of solution remains in the bottom of the tube. (This step may be hastened by pushing the tube deeper in the sand when only about 0.2 cc. of tan solution remains.)

The tube is then removed from the bath, 2 drops of concentrated HNO_3 are added, and the tube replaced in the sand bath. After the brown fumes of the oxides of nitrogen have evolved, the tube is pushed deeper into the sand to a point where the tube tip is at a temperature of over 250°. Digestion is allowed to continue for 2 to 4 hours, or overnight, after which time only a colorless drop of acid should remain in the tube. (If after an hour of this last digestion the solution is not colorless, another drop of HNO_3 should be added and heating continued.) The tube is then placed in a muffle furnace, and the temperature gradually raised to 400–500° and maintained at this temperature for 5 hours, after which only a white, ammonia-free ash remains in the tube.

A microburner flame can be used in the above ashing procedure instead of the sand bath. This will speed up the process, though the danger of splattering is increased and more actual working time is required.

[2] Made by the Macalaster-Bicknell Company, New Haven.

Chloroplatinate Precipitation—The ash is dissolved in 0.05 cc. of 20 per cent chloroplatinic acid in 1 N HCl by stirring with a fine glass rod, care being taken to dissolve all of the ash that may adhere to the wall of the tube. 2.0 cc. of absolute alcohol (saturated with K_2PtCl_6) is added, and the solution thoroughly agitated by means of the glass rod and allowed to stand for 20 minutes with occasional stirring. The adhering precipitate is then washed from the glass rod into the tube with 1.0 cc. of the absolute alcohol, but no attempt is made to mix this added alcohol with the rest of the solution, as this will cause a portion of the precipitate to rise on the wall of the tube. The tube is then centrifuged for 15 minutes at about 2000 R.P.M., after which the supernatant fluid is carefully poured off in one movement, the lip being quickly wiped with a clean, soft cloth. 1.0 cc. of the alcohol is now added, mixed with the glass rod, and the rod washed with 1.0 cc. of alcohol. Centrifugation is again carried out, and the washing and centrifugation again repeated. After the last wash alcohol has been poured off, the tube is allowed to drain for 30 seconds before the lip is wiped.

Color Development—The tube is placed in a water bath (85°). When all of the alcohol has evaporated, the temperature is lowered to 68° and the precipitate dissolved by stirring with the glass rod in about 3 cc. of hot distilled water. 1.0 cc. of hot 2 N potassium iodide is now added to the tube with stirring and the solution is allowed to stand at 68° for 10 minutes to develop the color fully. The tube is then cooled to room temperature by placing it in a bath of cold water, and the solution is washed into a 25 cc. volumetric flask with two 10 cc. portions of distilled water. The flask is filled to the mark, mixed by inversion, and the solution read in the colorimeter. Since the photelometer reading of the iodoplatinate solution varies somewhat with the temperature of the solution at the time of reading, all samples should be read at the same temperature as that used in determining the reference curve.

Calculations are made by reference to a curve obtained by the dilution of a known solution of potassium iodoplatinate. With each set of unknown plasma are run two standards, one containing a concentration of potassium near the lower limit of the suspected figure and the other near the upper limit of this figure. By plotting the known concentration of the standard solutions against the
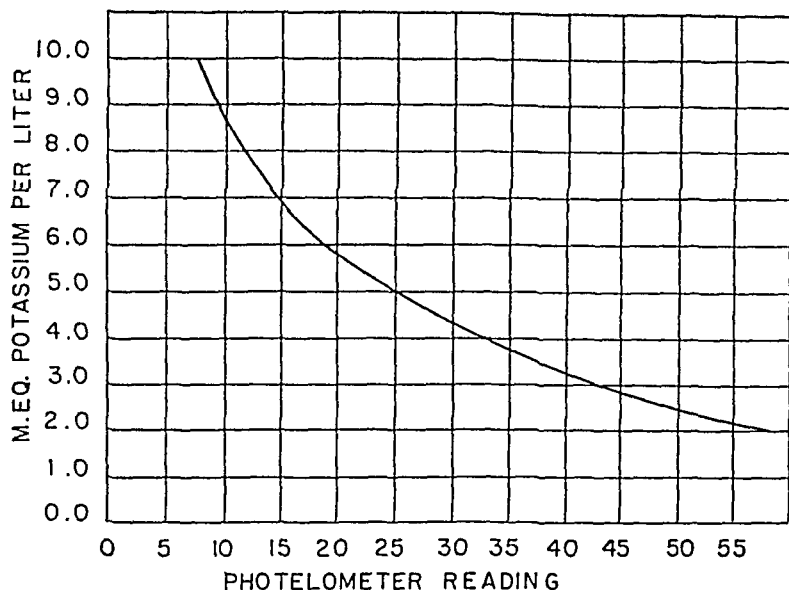
FIG. 1. The relationship between photelometer readings and concentration of potassium when 0.3 cc. of solution is analyzed.
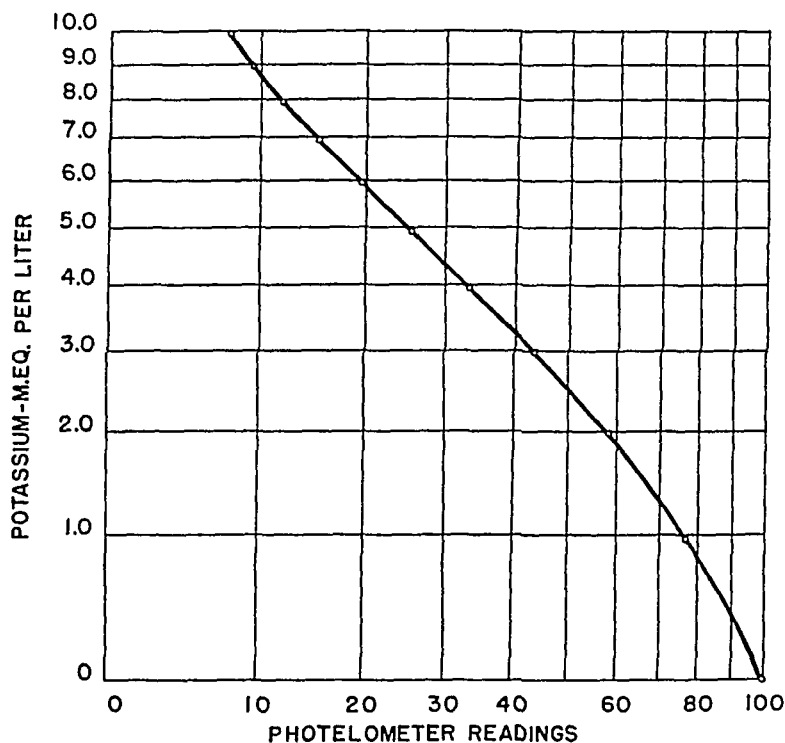
FIG. 2. Photoelectric curve obtained by plotting on logarithmic graph· paper the readings on varying concentrations of potassium iodoplatinate solution. The curve becomes a straight line in the concentrations between 3.0 and 8.0 milliequivalents per liter of potassium.

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determined concentrations, the correction for intermediate values can be obtained. The authors usually use standard solutions containing 4.91 and 7.35 milliequivalents per liter, the larger corrections appearing in the weaker solution. The standards must be carried through the entire ashing procedure, as the greatest error may occur during this part of the procedure.

Determination of Photelometer Curve—Slightly less than 30 mg. of K_2PtCl_6 (which has been purified and dried to constant weight) are weighed out accurately on a microbalance. This is dissolved in 100 cc. of hot distilled water in an Erlenmeyer flask, placed in a water bath at 68°, and 20 cc. of hot 2 N potassium iodide added. The flask is allowed to remain in the water bath for 10 minutes; the contents are then transferred with washing to a 1000 cc. volumetric flask and diluted to the mark with cool distilled water. This solution is then diluted one-tenth, two-tenths, three-tenths, etc., in 100 cc. volumetric flasks with distilled water. Portions from each flask are placed in matched colorimeter tubes and read. To obtain the number of milliequivalents per liter represented by the sample taken from the 1000 cc. flask, the number of mg. of K_2PtCl_6 in the weighed sample is divided by 2.917, and the concentration of the diluted standards then calculated from this figure. Fig. 1 shows the curve obtained by this procedure. Fig. 2 is the same curve plotted on logarithmic graph paper, showing that the curve becomes a straight line in the portion between 3.0 and 8.0 milliequivalents per liter, thereby following Beer's law.

Comment

Several variations in each step of the above technique have been investigated, and the results are worthy of comment.

Choice of Ashing Reagents—Shohl and Bennett (8) recommended 30 per cent H_2O_2 as an oxidizing agent. However, in all of the brands of the reagent tried in the present investigation 0.3 to 0.8 milliequivalent per liter of potassium was present. This gave higher readings which were not always constant owing to the need of adding the reagent by drops. Ashing with sulfuric acid alone, as proposed by Consolazio and Talbott (1), did not always give complete combustion. Sodium persulfate was tried, but an amount sufficient for complete oxidation of the plasma required

the addition of a disproportionate amount of sodium. Finally, the use of sulfuric and nitric acids alone with subsequent heating for 5 hours at 400–500° in a muffle furnace proved quite satisfactory in that ashing was complete, the ammonia driven off, and no potassium was added. Even by this method of ashing traces of potassium are lost through volatilization and probably also through solution in the glass. Much more potassium is lost when the ashing is carried out at 400° in Pyrex tubes than in quartz tubes at the same temperature. The procedure can be carried out in Pyrex tubes, but a much larger correction is required.

Precipitation—10 per cent chloroplatinic acid was tried, but the results on known solutions (which were dried but not ashed) were frequently 3 per cent low, a difficulty overcome by the use of a 20 per cent solution. The addition of sodium sulfate to the solution gives a larger precipitate which is more flocculent, adheres to the bottom of the tube, and does not rise on the wall of the tube. The use of sodium sulfate does not interfere with the final color.

Washing—All of the chloroplatinic acid must be removed from the tubes before the addition of the potassium iodide, as K_2PtCl_6 will form and interfere with the determination. Originally the supernatant wash fluids were removed by syphon suction; however, this has been found unnecessary with the use of sodium sulfate. With each washing the precipitate is broken up with a fine glass rod for each tube; these rods are hooked at the upper end and are hung on a rack during centrifugation, the same rod being returned to the same tube each time.

Color Development—All of the alcohol must be evaporated, as it will reduce the potassium iodoplatinate formed (8). As suggested by Shohl and Bennett (8), the color development may be hastened by the use of either heat or acid; however, on standing the acid causes a more rapid release of iodine, so that the solutions must be read in a very few minutes. By the use of heat the color remains relatively stable for several hours if the tubes are subsequently kept in a cool dark place.

Correction—As shown in Table I, unashed standard solutions may be read with an error of about 1 per cent; however, following the ashing procedure known solutions may be 0.3 milliequivalent per liter low in the weaker concentrations. This error may result from volatilization of potassium, solution of potassium in glass,

impure distilled water used in reagents, differences in solubility resulting from changes in the room temperature, and release of iodine in the final solutions.   However, in each set of determinations these factors are the same in all tubes and correction from standard solutions run with each set of determinations will give a final error of less than 3 per cent in the unknown plasma.   The figures given in Table II are the result of three sets of determinations and different corrections were needed for each set; yet the variation in the corrected concentration of the plasma was always less than 3 per cent.   The plasma used in these determinations was taken from a 3 day-old bank blood, and the per cent error is calculated from data on 10 cc. samples obtained by the gravi-

TABLE I

Determination of Varying Known Amounts of Potassium in Standard Solutions of Mixed Salts Equivalent to Inorganic Salt Concentration of Plasma

0.3 cc. samples were used.

Known concentration	Determined concentration	Error	
m.eq. per l.	*m.eq. per l.*	*m.eq. per l.*	*per cent*
4.91	4.95	+0.04	+0.8
6.55	6.63	+0.08	+1.2
8.19	8.25	+0.06	+0.7
9.82	9.85	+0.03	+0.3

metric method accepted by the Association of Agricultural Chemists (4).

The limiting factor in the number of determinations that can be run at one time is the capacity of the centrifuge.   The authors use an eight tube centrifuge head, and sixteen tubes are run at one time, the stirring and washing of half the tubes being carried out while the other half is in the centrifuge.   Unknowns are run in triplicate with two standard solutions in each group of eight tubes.

As indicated by Shohl and Bennett (8), care must be taken throughout the entire procedure to keep ammonia from the solutions, since it also forms an insoluble chloroplatinate which gives a similar color on the addition of potassium iodide.

TABLE II

Determination of Potassium on Sample of Plasma from Bank Blood

10 cc. gravimetric determinations in triplicate gave a concentration of 7.40 milliequivalents per liter.

The high potassium value is due to the fact that the plasma was taken from stored blood after some potassium had diffused from cells to plasma.

Sample No. (0.3 cc.)	Potassium*	Error	
	m.eq. per l.	*m.eq. per l.*	*per cent*
1	7.55	+0.15	+2.0
2	7.55	+0.15	+2.0
3	7.35	−0.05	−0.7
4	7.35	−0.05	−0.7
5	7.35	−0.05	−0.7
6	7.25	−0.15	−2.0
7	7.55	+0.15	+2.0
Average	7.42	+0.02	+0.4
8	7.50	+0.10	+1.4
9	7.35	−0.05	−0.7
10	7.45	+0.05	+0.7
11	7.50	+0.10	+1.4
12	7.40	0.00	0.0
13	7.45	+0.05	+0.7
Average	7.44	+0.04	+0.5
Samples of above plasma diluted to concentration of 4.93 milliequivalents per liter of potassium			
1	4.84†	−0.09	−1.8
2	4.89†	−0.04	−0.8
3	4.82†	−0.11	−2.2
4	4.84†	−0.09	−1.8
5	4.84†	−0.09	−1.8
Average	4.85	−0.08	−1.6

* Correction from standards, Samples 1 to 7 = −0.35 milliequivalent per liter; Samples 8 to 13 = +0.30.

† Correction from standards = −0.09 milliequivalent per liter.

Owing to the high concentration of potassium in blood cells, care must be taken to prevent hemolysis before separation of the plasma. Also, since potassium rapidly diffuses from cells to

plasma on standing, the separation should be carried out immediately after the blood is taken. Scudder (6) has shown that ammonia increases the speed of this diffusion. The present authors use blood taken into dry heparin, and centrifugation is started within 5 minutes after it is drawn.

This method has been used also for the determination of potassium in blood serum, whole blood, urine, bile, feces, and food. Samples should be used which contain between 0.03 and 0.30 mg. of potassium; and, if the final dilution to 25.0 cc. gives a con-

TABLE III

Potassium Balance Study on Patient with Continuous Intestinal Suction by Means of Miller-Abbott Tube

The tip of the tube was lying in the terminal ileum.

Potassium values are expressed as mg. per 24 hours.

Days of study	K intake			K output				Total intake minus total output
	Intravenous	Food	Total intake	Suction drainage	Feces	Urine	Total output	
1	1000	750	1,750	120	0	810	930	+820
2	0	940	940	200	0	860	1,060	−120
3	0	1,200	1,200	74	0	1,180	1,254	−54
4	1000	1,000	2,000	165	380	990	1,535	+465
5	0	1,360	1,360	70	0	1,160	1,230	+130
6	0	1,340	1,340	650	80	1,280	2,010	−670
7	0	1,350	1,350	81	0	1,570	1,651	−301
8	0	1,070	1,070	47	0	1,550	1,597	−527
9	0	1,220	1,220	62	0	1,000	1,062	+153
Totals...	2000	10,230	12,230	1469	460	10,400	12,329	−99

centration higher than the upper limit of the curve, the solution should be further diluted to 50 or 100 cc. in volumetric flasks, a correction being made for the degree of dilution. Complete intake-output studies of potassium have been carried out by this method on subjects in which intestinal suction was established with the Miller-Abbott tube, and balances were obtained over as long as a 9 day period of study (5). Table III shows the result of one such balance study on a patient whose tube extended to the terminal ileum. The intravenous potassium intake was in the form of whole blood, estimated to contain 2000 mg. of potassium per liter. All figures are given as mg. of potassium per 24 hours.

As a further means of studying this method, the authors have investigated the changes in the plasma potassium of stored blood. Scudder, Drew, Corcoran, and Bull (7), DeGowin, Harris, and Plass (2), and others have demonstrated a diffusion of potassium from cells to plasma in preserved blood; in the present study their findings have been confirmed.   The technique of study consisted of taking 450 cc. of blood from a human donor into 75 cc. of 2.5
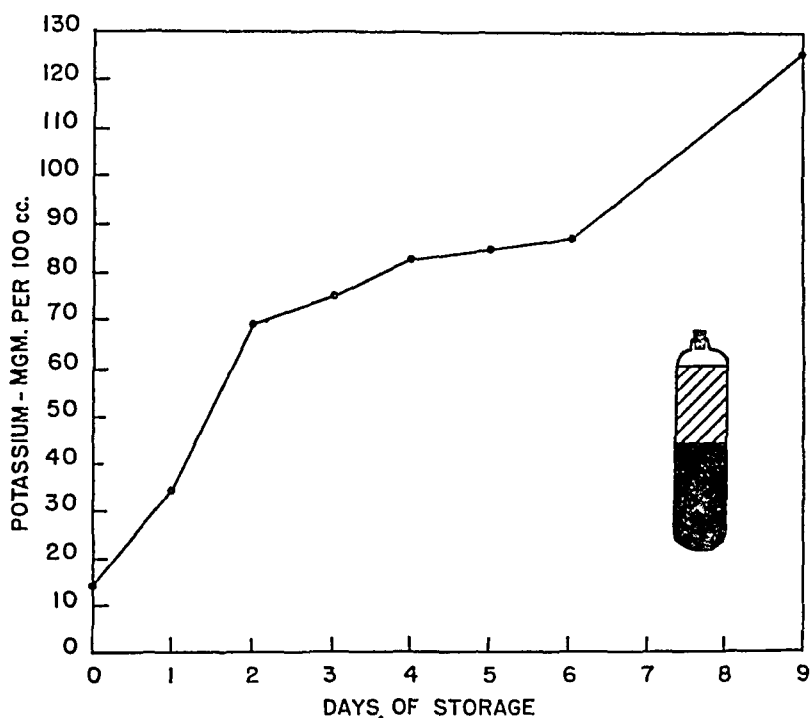
Fig. 3. The rise in plasma potassium of stored blood.   The type of bottle used for storage is illustrated.

per cent sodium citrate; this was mixed and immediately divided into 40 cc. storage bottles with sterile precautions.   The diameter of the bottles was 3.0 cm.; about 3 cc. of air remained above the blood which was kept tightly stoppered.   The bottles were then allowed to stand in an ice box at 5°.   On the days of sampling one bottle was removed, tipped three times to mix cells and plasma, and then centrifuged at 2000 R.P.M. for 30 minutes.   The supernatant plasma was then removed and the potassium content determined.   Fig. 3 shows the result of one such study in which the

potassium level rose from 14 mg. per 100 cc. of plasma at the time the blood was taken to 127 mg. at the end of 9 days. In this particular experiment visible hemolysis was not present until the 9th day of storage.

SUMMARY

A procedure is presented which adapts the chloroplatinic acid precipitation method for potassium to 0.3 cc. samples of plasma, the final reading being based upon the photoelectric determination of potassium iodoplatinate with a correction made from known standards carried through the procedure with the unknown solution. The method has been used in the study of blood preservation; it is also adaptable to other biological materials and has been used in following the potassium balance in certain patients.

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MICROMETHODS FOR THE DETERMINATION OF SPHINGOMYELIN AND CHOLINE

APPLICATIONS FOR THE ESTIMATION OF THE PHOSPHOLIPID PARTITION (SPHINGOMYELIN, LECITHIN, AND CEPHALIN) IN BLOOD AND TISSUES[*]

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A micromethod for the determination of sphingomyelin and modifications of the choline enneaiodide micromethod have been developed. These procedures, together with the analysis of the total phospholipid, provide a means of determining the individual phospholipids, lecithin, cephalin, and sphingomyelin, in small amounts of blood and tissues, as outlined in Table I.

The use of Reinecke salt $Cr(NH_3)_2(SCN)_4NH_4$ as a specific precipitating agent for sphingomyelin has been employed by Thannhauser and his group (2, 3) in the development of a macrogravimetric method, which has been adapted for microprocedure. Rather than weighing the sphingomyelin reineckate precipitate, $C_{45}H_{91}N_8PO_7S_4Cr$, we determine the phosphorus content, from which sphingomyelin is calculated.

Modifications of Roman's choline micromethod (4, 5) include the use of immersion filter sticks which facilitate washing the labile choline enneaiodide precipitate $C_2H_5(CH_3)_3NOI_9$ with minimum disturbance at the temperature of melting ice, and the incorporation of a technique for the conversion of iodide to iodate with bromine (6) preceding titration with standard sodium thiosulfate.[1] The latter modification offers the advantages of ready

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[*] Presented before the Ninety-seventh meeting of the American Chemical Society, Division of Biological Chemistry, at Baltimore, April 3–7, 1939.

[1] $XI + Br_2 \rightarrow XBr + IBr$; $IBr + 2Br_2 + 3H_2O \rightarrow HIO_3 + 5HBr$;

solubility of the precipitate in the bromine solution and a 6-fold increase in the final titration value.[1]

Lipid Extraction Methods—Analyses can be made directly on alcohol-ether (7) or chloroform-methanol (3) extracts, or on petroleum ether reextracts (8). Evidence has accumulated, however, that the phospholipids are not completely reextracted with petroleum ether (9–11), although recovery is far more complete when evaporation of the alcohol-ether is carried out under carefully

TABLE I

Determination of Phospholipid Partition (Lecithin, Cephalin, Sphingomyelin)

		Size of sample necessary for triplicate determinations			
	Method of determination	Plasma	Red blood cells	Stroma (dry weight)	Brain (wet weight)
		cc.	*gm.*	*mg.*	*mg.*
Total phospholipid	Phosphorus content × 23.54	0.5	0.3	5	15
Choline phospholipid	Molecular ratio of choline to phosphorus × total phospholipid, or choline × 6.65 (approximate)	1.5	1.5	60	150
Sphingomyelin	Phosphorus of sphingomyelin reineckate × 25	1.5	1.5	60	100
Cephalin*	Subtraction of choline phospholipid from total phospholipid				
Lecithin	Subtraction of sphingomyelin from choline phospholipid				

* Cephalin can also be determined from the amino nitrogen of lipid extracts free of contaminating nitrogen constituents (1).

controlled conditions, in nitrogen, under reduced pressure, and at temperatures not exceeding 50° (9–11). Unpublished data from this laboratory[2] have indicated that the type of phospholipid

$$HIO_3 + 5KI \rightarrow 3I_2; \quad 3I_2 + 6Na_2S_2O_3 \rightarrow 3Na_2S_4O_6 + 6NaI; \quad I = \frac{HIO_3}{6} = Na_2S_2O_3.$$
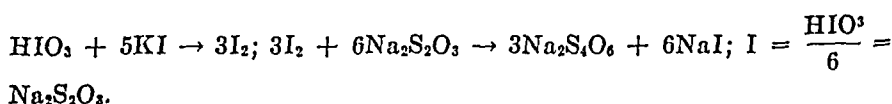
[2] Data from a thesis submitted by Ira Avrin in partial fulfilment of the requirements for the degree of Master of Science from the Department of Chemistry, Wayne University, Detroit (1937).

present, as well as oxidative changes, may account for low recoveries. Diminished recoveries of phosphorus were secured with increased exposure times of the dried alcohol-ether extracts to air, with lower fatty acid carbon to phosphorus ratios of the precipitated phospholipids (the theoretical ratios for lecithin, cephalin, and sphingomyelin are 13.9, 13.9, and 7.4, respectively). Data recently secured (Table II) show that both lecithin and sphingomyelin are refractory to petroleum ether extraction, whereas cephalin is extracted completely.

These observations emphasize the necessity of employing solvents which extract all the phospholipids, *i.e.* alcohol-ether or

TABLE II

Determination of Individual Phospholipids in Alcohol-Ether, Petroleum Ether, and Treated Alcohol-Ether* Extracts

Human blood	Plasma, mg. per 100 cc.			Cells, mg. per 100 gm.	
	Alcohol-ether extract	Petroleum ether re-extract	Treated alcohol-ether extract*	Alcohol-ether extract	Petroleum ether re-extract
Total phospholipid..............	216	162	200	344	305
" choline phospholipid.........	138	70	128	114	76
Lecithin.......................	115	60	105	51	43
Sphingomyelin...................	23	10	23	63	33
Cephalin (by difference)..........	78	92	72	230	229
" (from amino N)...........			81		

* Used according to the procedure of Folch and Van Slyke (1) to remove contaminating nitrogen constituents.

chloroform-methanol, particularly in studying samples of pathological blood.[3] A procedure recently developed by Folch and Van Slyke (1) yields alcohol-ether extracts which are free of contaminating nitrogen constituents and eliminates any necessity for reextraction with petroleum ether. Preliminary analyses of individual phospholipids by methods presented herein demonstrate excellent agreement with values on untreated alcohol-ether extracts (Table II); furthermore, corresponding values for cephalin

[3] With petroleum ether lowered recoveries of alcohol-ether-soluble phosphorus compounds have been observed in certain pathological blood samples (pernicious and hemolytic anemias), indicating an abnormal phospholipid mixture (see foot-note 2).

determined directly from the amino nitrogen and indirectly by difference check fairly closely (Table II).[4]

Method for Determination of Sphingomyelin

Apparatus—Combustion tubes and Van Slyke-Neill manometric apparatus (1, 8–12); phosphorus digestion tubes with constriction at the 25 ml. mark;[5] Jena sintered glass immersion Filter 94G3 with 0.3 inch base and capillary stem.

Solutions—Absolute methyl alcohol, concentrated hydrochloric acid, ethyl ether, petroleum ether, acetone, saturated solution of Reinecke salt[6] in absolute methyl alcohol.

Procedure—Suitable aliquots (Table I) of the lipid extract are measured into titer tubes and evaporated gently on a water bath, with caution to avoid heating or standing after the samples become dry. Each residue is dissolved in 1 cc. of absolute methyl alcohol; then 1 cc. of an acidified (1 drop of concentrated HCl per cc.) saturated solution of Reinecke salt in methyl alcohol is added. The tubes are stoppered and chilled in a refrigerator overnight. The following morning the tubes containing the sphingomyelin reineckate which has precipitated in the cold are placed in an ice bath and washed with six 1 cc. portions of ice-cold methyl alcohol and then with six 1 cc. portions of cold ethyl ether to remove contaminating phospholipids.[7] The supernatant fluid and washings are removed with a small sintered glass immersion filter, which facilitates washing the precipitate while the tube is in the ice bath.

The precipitate is dissolved in 5 cc. of a 1:1 mixture of acetone and methyl alcohol, heated to boiling (by immersion of the tube

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[4] Corresponding values on a purified alcohol-ether extract of cells, as determined from the amino nitrogen and by difference, were 172 and 184 mg., respectively.

[5] May be procured from the Scientific Glass Apparatus Company.

[6] Reinecke salt may be purchased from the Eastman Kodak Company or Eimer and Amend.

[7] The recent modification of the macrogravimetric procedure (13) in which the reineckate precipitate is washed also with cold acetone did not alter the phosphorus content. Corresponding values for the phosphorus content of precipitates washed with acetone in addition to methanol and ethyl ether were, respectively, 0.00938 and 0.00936 mg., 0.00874 and 0.00883 mg., 0.00515 and 0.00515 mg.

in a beaker of boiling water), and then drawn off through the filter stick into a phosphorus digestion tube with a 25 ml. graduation in the constricted area (Fig. 1). Three additional washings are made with 5 cc. portions of the solvent mixture, which are then heated and similarly drawn off into the digestion tube. The solution is then evaporated to dryness on a water bath. Digestion, precipitation, and gasometric determination of the phosphorus are carried out according to the procedures of Kirk
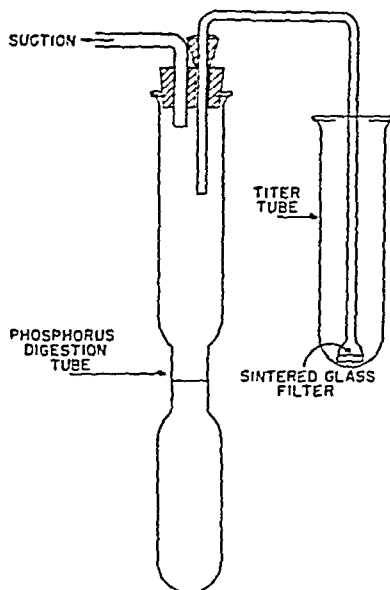
FIG. 1. Apparatus for transferring the sphingomyelin precipitate to the digestion tube.

(14) and Van Slyke, Page, and Kirk (12). (If the equipment for gasometric analysis is not available, the phosphorus may be determined by any standard micromethod.) A blank determination for phosphorus with all of the reagents should be made simultaneously. The phosphorus content multiplied by the factor 25 gives the sphingomyelin content of the sample.

Results—Analyses of standard sphingomyelin samples[3] within

[3] Phospholipids were isolated from alcohol-ether extracts of erythrocyte stroma and brain and subjected to procedures for removing lecithin,

a range of 0.24 to 0.554 mg. are given in Table III. The data demonstrate complete recoveries of as little as 0.1 mg., which approaches the limits of accuracy of the phosphorus micromethod. When standard samples of sphingomyelin were added to lipid extracts of erythrocyte stroma, recoveries of 96 to 110 per cent with an average of 102 per cent were secured in the presence of

TABLE III

Recovery of Sphingomyelin

	Present	Added*	Total present	Total recovered		Other† phospholipid present
	mg.	*mg.*	*mg.*	*mg.*	*per cent*	*mg.*
Sphingomyelin	0.024		0.024	0.021	88	
preparations*	0.047		0.047	0.036	76	
	0.094		0.094	0.106	113	
	0.139		0.139	0.110	79	
	0.142		0.142	0.143	101	
	0.189		0.189	0.183	97	
	0.277		0.277	0.289	104	
	0.277		0.277	0.266	96	
	0.554		0.554	0.543	98	
	0.554		0.554	0.563	102	
Stroma extracts	0.079	0.008	0.087	0.088	101	Not determined
	0.212	0.008	0.220	0.226	103	" "
	0.079	0.215	0.294	0.314	107	" "
	0.139	0.189	0.327	0.317	97	" "
	0.277	0.094	0.371	0.361	97	0.08
	0.107	0.277	0.384	0.377	98	0.60
	0.277	0.142	0.419	0.415	99	0.16
	0.114	0.322	0.436	0.419	96	Not determined
	0.554	0.024	0.578	0.580	100	3.00
	0.554	0.047	0.601	0.661	110	1.80
	0.554	0.107	0.661	0.713	108	0.60

* Phospholipids isolated from brain and erythrocyte stroma (see footnote 8).

† Lecithin and cephalin.

large amounts of lecithin and cephalin (Table III). This method applied to samples of brain, blood plasma, and cells has given

cephalin, cerebrosides, and other lipids, as outlined by Thannhauser and Setz (2). Preparations of varying purity were obtained (70 to 90 per cent sphingomyelin as shown by carbon, nitrogen, and phosphorus analyses) and used for the standard sphingomyelin solutions.

results which agree within 4 per cent and correspond in magnitude with those determined by the macrogravimetric procedure (13, 15) (Table VI).

Method for Determination of Total Choline Phospholipids

Apparatus—Titer tubes, 1 by 4 inches; conical tipped centrifuge tubes, 60 cc. capacity; alundum immersion filter sticks with Filter RA225.[5]

Solutions—Ethyl alcohol, 95 per cent; barium hydroxide, saturated; phenol red, aqueous, 0.4 per cent; hydrochloric acid, 10 per cent; sodium acetate, 25 per cent; formic acid, 90 per cent; potassium iodide, 10 per cent (freshly prepared); starch, 1 per cent; iodine-potassium iodide reagent, 15.7 gm. of iodine and 20 gm. of potassium iodide in 100 cc. of water; bromine solution, 10 per cent potassium acetate in glacial acetic acid plus 6 drops of bromine per 100 cc.; standard sodium thiosulfate, 0.02 N, from which 0.005 N solution is freshly prepared by dilution.

Procedure.   Hydrolysis with Barium Hydroxide—A method for determination of choline, in which barium hydroxide is used for hydrolysis of choline phospholipids, has been reported from this laboratory (11). It has been reported recently, however, that barium hydroxide brings about only a partial splitting of sphingomyelin, whereas a new technique in which gaseous HCl in absolute methanol is employed effects complete hydrolysis (13). For this reason, a comparative study was made of the two hydrolytic procedures on small amounts of phospholipid such as are required for the choline microdetermination which will be discussed later in this paper.

Certain modifications of the original barium hydroxide procedure have been incorporated.   10 cc. of ethyl alcohol and 5 cc. of saturated barium hydroxide are added to suitable aliquots (Table I) of the lipid extract in titer tubes.   The tubes are heated on the steam bath until the contents are reduced to a volume of approximately 1 cc.; the sides are then washed with an additional 10 cc. of ethyl alcohol and the tubes again heated until the volume is reduced to 0.5 to 1 cc.   Caution must be exercised to prevent them from becoming completely dry.   The residue is made slightly acid with 10 per cent HCl in the presence of phenol red as indicator, and filtered through a small funnel (3 cm., No. 1

paper) into a 50 cc. conical centrifuge tube. The titer tube is washed thoroughly with several 1 cc. portions of water which are filtered into the centrifuge tube; the final volume should not exceed 10 to 15 cc. It was found unnecessary to extract the hydrolysate with petroleum ether, for the filtration completely removed the fatty acid residue (11).

Hydrolysis with HCl-Methanol—The HCl-methanol procedure for hydrolysis of the choline phospholipids (13) was adapted to microprocedure. Suitable aliquots of lipid extract were evaporated to dryness in conical tubes with 24/40 ground glass joints and then refluxed for 3 hours under either air or water condensers fitted with 24/40 joints. The hydrolysate was evaporated to dryness *in vacuo* (by means of a vacuum condenser equipped with similar interchangeable ground glass connections), taken up in 1 cc. of water, and acidified with 10 per cent HCl. Removal of the finely suspended fatty acid residue was attempted by both centrifugation (13) and filtration as in the foregoing procedure, but the solutions remained turbid, which interfered to some extent with the precipitation of choline. In addition to the possibility of carrying down absorbed iodine, the precipitate was deposited along the sides of the tube rather than in a compact mass at the tip, thus making filtration and washing difficult. Other analyses were made upon clear solutions secured by extracting the hydrolysate with petroleum ether before filtration; however, the choline values obtained on these solutions were lower than those for the barium hydroxide hydrolysates after extraction with petroleum ether (Table V).

Precipitation of Choline—The centrifuge tubes containing the hydrolysates are chilled in an ice-salt bath to a temperature of 5–10° before the addition of 0.4 cc. of iodine-potassium iodide reagent for each cc. of solution. After addition of the reagent the tubes are allowed to stand in the ice bath for 30 minutes, then centrifuged at high speed for 15 minutes, and replaced in the ice-salt bath. The supernatant fluid and wash solutions are removed with alundum immersion filter sticks, which enables washing the labile precipitate with minimum losses from volatilization. The precipitate is washed with 1 cc. portions of water (5–10°) until the wash solution is colorless (usually five washings suffice), with caution to avoid disturbing or sucking the precipitate

onto the filter. The filter stick is allowed to remain in the tube while 5 cc. of the bromine solution are added. It is advisable to stir up the precipitate with the filter and then allow the tubes to stand for several hours for complete conversion of the iodide to iodate. The filter sticks are then washed (water being forced through the filter to rinse the inside) and removed.

TABLE IV

Recovery of Choline

	Present	Added*	Total present	Total recovered	
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
Choline standard*	0.044		0.044	0.035	80
	0.087		0.087	0.065	75
	0.131		0.131	0.137	104
	0.174		0.174	0.174	100
	0.174		0.174	0.164	94
	0.174		0.174	0.172	99
	0.211		0.211	0.205	97
	0.211		0.211	0.214	101
	0.211		0.211	0.201	95
	0.211		0.211	0.219	104
	0.261		0.261	0.241	92
Brain extracts	0.212	0.049	0.261	0.264	101
	0.212	0.049	0.261	0.253	97
	0.212	0.098	0.310	0.333	107
	0.212	0.098	0.310	0.335	108
	0.085	0.244	0.329	0.356	108
	0.085	0.244	0.329	0.329	100
	0.162	0.244	0.406	0.388	96
	0.162	0.244	0.406	0.414	102
	0.212	0.211	0.433	0.418	96
	0.212	0.211	0.433	0.454	105
	0.212	0.211	0.433	0.463	107

* Choline was added as standard choline hydrochloride solutions which were checked by nitrogen, carbon, and chloride determinations.

Just before titration, 2 cc. of a 25 per cent sodium acetate solution and 10 drops of formic acid are added. This should decolorize the solution completely. (Should a yellow color persist, indicating incomplete oxidation of the iodine, more bromine solution should be added and the solution allowed to stand again.) 5 cc. of a freshly prepared 10 per cent potassium iodide solution are

added, followed by immediate titration with standard 0.005 N sodium thiosulfate, the end-point being adjusted in the presence of 1 cc. of a 1 per cent starch solution. Blank determinations on acidified water in place of the hydrolysate solution should be made simultaneously and subtracted from the titration values.

Calculation—1 cc. of 0.005 N sodium thiosulfate is equivalent to 0.01122 mg. of choline. The molecular ratio of choline to phosphorus multiplied by the mg. of total phospholipid gives the

TABLE V

Comparison of Hydrolytic Procedures in Recovery of Choline from Brain Extracts

Brain extract sample No........	Choline recovered from extracts,* *mg.*							
	I		II		III		IV	
Method of hydrolysis...........	HCl-meth-anol	Bari-um hy-drox-ide	HCl-meth-anol	Bari-um hy-drox-ide	HCl-meth-anol	Bari-um hy-drox-ide	HCl-meth-anol	Bari-um hy-drox-ide
Analysis of samples after hydrolysis	0.358	0.357	0.250	0.212	0.213	0.212	0.165	0.162
Analysis of samples + 0.211 mg. choline after hydrolysis			0.429	0.445	0.255†	0.343†		
Analysis of samples + 0.244 mg. choline after hydrolysis							0.411	0.401
Analysis of samples after hydrolysis and reextraction with petroleum ether	0.301	0.374			0.128		0.081	

* All values represent averages of three determinations.
† Two-fifths of the sample + 0.211 mg. of choline.

mg. of choline phospholipid (11). Multiplication of the mg. of choline by the factor 6.65 yields a less accurate value (11).

Results—Representative data on the recovery of choline from standard choline hydrochloride solutions are given in Table IV. Complete recovery can be obtained with samples equivalent to 0.15 mg. or more of choline. Recoveries of 92 to 108 per cent were secured from standard samples added to lipid extracts of brain and subjected to barium hydroxide hydrolysis.

Comparisons between barium hydroxide and HCl-methanol hydrolyses of brain extracts are given in Table V. Practically identical values for choline were found by the two hydrolytic procedures in three of four series of determinations (in which approximately 25 per cent of the total phospholipid was present as sphingomyelin). With phospholipid preparations which were more than 50 per cent sphingomyelin, choline values higher by 10 per cent were obtained with the HCl-methanol method. Analyses of sheep (56 per cent sphingomyelin) and dog brains

TABLE VI

Lecithin, Cephalin, and Sphingomyelin Partition of Total Phospholipids

		Total phospho-lipid	Total choline phospho-lipid		Lecithin		Cephalin		Sphingo-myelin	
		mg.	mg.	per cent*	mg.	per cent*	mg.	per cent*	mg.	per cent*
Plasma, per 100 cc.	Micro†	189	134	71	99	52	55	29	35	19
	Macro‡	226	130	57	107	47	96	43	23	10
	Micro§	145	77	53	19	13	68	47	58	40
Erythrocytes, per 100 gm.	Micro†	317	127	40	77	24	190	60	50	16
	Micro§	196	79	40	32	16	117	60	47	24
Stroma, per 100 mg. dry weight	Micro‖	10	5	50	2	20	5	50	3	30
Brain, per 100 mg. dry weight	Micro¶	20	8	40	4	20	12	60	4	20
	Macro‡	31	11	35	5	16	20	65	6	19

* Per cent of total phospholipid.
† Average of four samples, human.
‡ Values from Thannhauser and group (13, 15).
§ Approximate; values from Kirk (16).
‖ Average of three samples, human.
¶ Average of four samples, dog.

(51 per cent sphingomyelin) produced average choline phospholipid values of 70 and 75 per cent, respectively, with the HCl-methanol method; the corresponding values with the barium hydroxide method were 61 and 65 per cent, respectively.

From the data it appears that practically complete hydrolysis is effected by barium hydroxide with the amounts of lipid used for the choline microdetermination. This is further substantiated by the close correspondence of reported choline phospholipid values for blood serum on the basis of HCl-methanol hydrolysis

and choline macroprocedures (13) with values determined by the micromethods for hydrolysis and choline presented in this study (Table VI).

Preliminary data on the distribution of the individual phospholipids in normal human blood plasma, erythrocytes, and erythrocyte stroma, as well as in dog brain are given in Table VI, together with analyses reported on the basis of macroprocedures (13, 15) and approximate micromethods (4, 16). Similar comparative values for total choline phospholipids, lecithin, and sphingomyelin in plasma are shown between the results secured by the macro- and micromethods presented here. We have found lower values for total phospholipid and cephalin but have demonstrated fair agreement with values for cephalin determined directly from the amino nitrogen on purified alcohol-ether extracts (see Table II and accompanying discussion). The analyses reported by Kirk for the approximate estimation of individual phospholipids (16) demonstrate lower values for total phospholipid, choline phospholipids, and particularly lecithin, which may be due to evaporation of the alcohol-ether extracts in air. The present data showing the susceptibility of the choline phospholipids to oxidative changes which affect their solubility emphasize the criticism which has been made (13) of the indirect determination of sphingomyelin by differential solubility of the phospholipids in ethyl ether (4, 16).

Characteristic patterns for the distribution of individual phospholipids in plasma, red cells, and brain are evident. Over half of the total phospholipid of erythrocytes consists of cephalin, with the remainder about equally distributed as lecithin and sphingomyelin. In contrast, lecithin constituted about 50 per cent of the plasma phospholipids, cephalin a fair proportion (30 to 40 per cent), and sphingomyelin the least. The phospholipid partition of brain tissue is similar to that of erythrocytes, with cephalin comprising 60 per cent of the total, and the remainder equally divided as lecithin and sphingomyelin.

Studies on the phospholipid distribution in blood, stroma, and brains of dogs with experimental anemia due to n-propyl disulfide are reported elsewhere (17). Observations are in progress on the

blood phospholipid changes in pernicious anemia and polycythemia and on the stroma of erythrocytes in certain pathological conditions.

SUMMARY

A micromethod for the determination of sphingomyelin and modifications of the choline enneaiodide micromethod have been developed. These procedures together with total phospholipid determination provide a means of determining the individual phospholipids, lecithin, cephalin, and sphingomyelin, in small amounts of blood and tissues.

Data are presented from a comparative study of the barium hydroxide and HCl-methanol hydrolytic procedures for the liberation of choline from the choline-containing phospholipids (lecithin and sphingomyelin).

Other data show the lability of choline phospholipids to oxidative changes and their refractoriness to petroleum ether extraction, thus emphasizing the necessity of employing lipid solvents which extract all of the phospholipids.

Results of preliminary analyses on blood plasma, cells, stroma, and brain demonstrate characteristic distribution patterns of the individual phospholipids.

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SYNTHESIS OF FACTOR V (PYRIDINE NUCLEOTIDES) FROM NICOTINIC ACID IN VITRO BY HUMAN ERYTHROCYTES

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We have shown that incubation of defibrinated human blood with nicotinic acid leads to a marked increase in the factor V[1] content of the cells, and that ingestion of nicotinic acid by man leads to a similar increase in the factor V content of the blood cells (1). We believed that the synthesis occurred in the erythrocytes, because the factor V content of normal blood is practically confined to them. Axelrod, Gordon, and Elvehjem (2), who recently confirmed the synthesis *in vivo*, have also taken the same view. Vilter, Vilter, and Spies (3), however, although able to confirm the synthesis *in vitro* by blood cells, state that "...it seemed that the red cells stored and carried the enzymes instead of performing the synthesis." This statement was based on their inability to demonstrate synthesis with erythrocytes washed free of leucocytes. They concluded that, "The above findings are inconsistent with the statement of Kohn and Klein that the normal erythrocytes accomplish the synthesis ... and suggest the tentative hypothesis that nucleated cells are essential for the synthesis ..."

The object of this work is to reinvestigate the synthesis of factor V by erythrocytes.

EXPERIMENTAL

To prove that erythrocytes can synthesize factor V *in vitro* it is necessary to show that the amount of synthesis by a suspension

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[1] Factor V is the factor necessary for the cultivation of *Hemophilus parainfluenzae*. Of known compounds only di- and triphosphopyridine nucleotide can serve as factor V.

of blood cells is independent of the number of leucocytes present. Obviously, it is essential to use a suspension of undamaged cells. Before gross injury such as hemolysis is evident, the ability of the erythrocytes to synthesize factor V can be decreased by washing with an unfavorable medium and undue handling; *e.g.*, too frequent passage through capillary pipettes. The level of factor V can
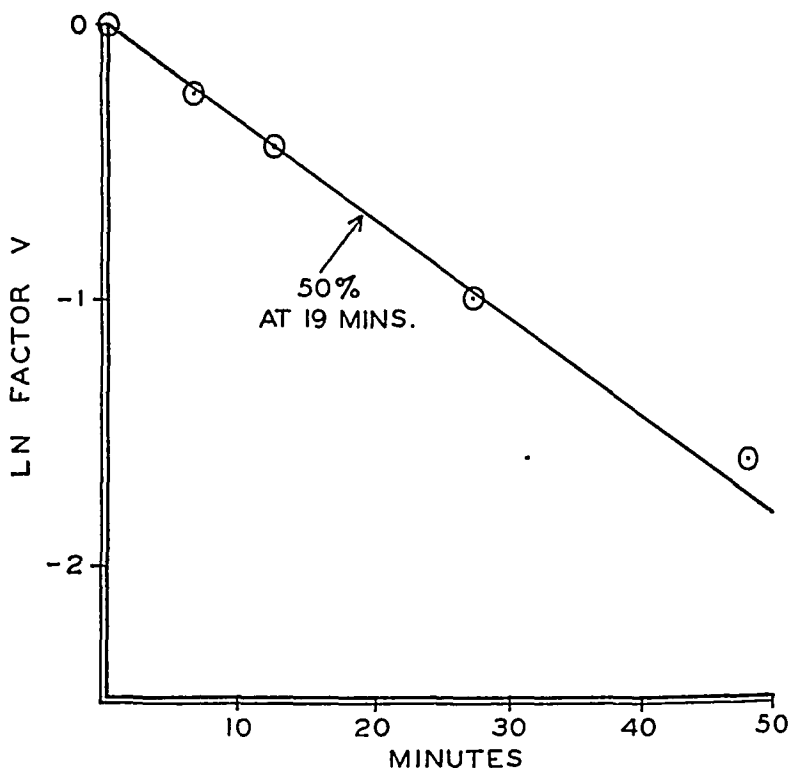
FIG. 1. Disappearance of factor V from blood following hemolysis, at 25°. 1 ml. of venous blood was mixed with 9 ml. of water, and samples were assayed for factor V at various times thereafter. The rate of inactivation, which is first order with respect to time, is due chiefly to the heat-labile system liberated from the cells by hemolysis.

also be decreased by such treatment. These effects may be due in part to the release of a heat-labile system within the cells which destroys factor V. The existence of such a system may be demonstrated by hemolyzing the cells in water and following the progressive loss of factor V, as shown in Fig. 1. The technique of the factor V assay has been previously described (4, 5).

The following experiment illustrates how the synthesizing abil-

ity of the cells may be damaged by the use of unsuitable suspension media. Venous blood was defibrinated by shaking with glass beads. Two 1 ml. aliquots were placed in stoppered test-tubes and kept at room temperature during the preparation of the following cell suspensions. Four samples of the blood were centrifuged 10 minutes at 2400 R.P.M. The supernatant liquid was removed. One sample of cells was mixed by gentle pipetting with an equal volume of sodium chloride solution, 0.9 gm. per 100 ml., a second with Ringer-phosphate solution, 0.025 M phosphate, pH 7.4, a third with Ringer-phosphate solution containing 1 mg. of glucose per ml. In these cases care was taken not to remove the white cells. In the fourth case about two-thirds of the cells was removed from the bottom of the tube with a pipette whose tip was drawn out to a long capillary, transferred to a dry tube, and mixed with an equal volume of Ringer-phosphate solution containing glucose. The suspensions were centrifuged 8 minutes. The supernatant liquid was removed and the cells treated as before, with 4 volumes of medium. The suspensions were then centrifuged 5 minutes, the supernatant liquid removed, and the cells treated as before, with 1.5 volumes of medium. These operations were carried out under sterile conditions over a 5 hour period.

1 ml. of each suspension plus 0.05 mg. of neutralized nicotinic acid in 0.1 ml. of water and, as a control, 1 ml. of each suspension plus 0.1 ml. of its suspending medium were incubated 19 hours at 33–35°. After incubation, during which no detectable hemolysis occurred, the factor V in each mixture was assayed. The percentage increase in factor V in each mixture containing nicotinic acid over its control was calculated as a measure of synthesis. The results are given in Table I.

The data in Table I show that the synthesizing ability of the cells is greater in Ringer-phosphate solution containing glucose than in Ringer-phosphate or sodium chloride solution.

The increase in factor V in the case of the preparation made from the lower layer of cells was 27 per cent. The decrease in leucocytes was 99.5 per cent; the decrease in synthesis of factor V with respect to its similarly washed control was 44 per cent. That the decrease in factor V synthesis was not a function of the decrease in white cells, but of the manipulations employed in preparation, is shown by the experiment given below.

Venous blood was defibrinated by shaking with glass beads. Two 1 ml. aliquots of the blood were placed in stoppered tubes and kept at room temperature during the preparation of the following suspensions. In order to remove the leucocytes 8 ml. of blood were mixed with 5 ml. of Ringer-phosphate solution containing glucose, and then centrifuged 5 minutes at 2400 R.P.M. The upper layer of cells and the supernatant liquid were removed, leaving 4 ml. of cells which were mixed with 8 ml. of medium and centrifuged for 5 minutes. The upper layer of cells and supernatant liquid were removed, leaving 2.5 ml. of cells which were finally suspended in 2.5 ml. of medium. There were ten leucocytes per million erythrocytes in this preparation. Another sample of

TABLE I

Effect of Suspension Medium upon Synthesis of Factor V by Human Blood Cells

Medium	Per cent increase in factor V	Leucocytes per million erythrocytes
Serum..	68	1000
Sodium chloride solution.......................	18	1000
Ringer-phosphate solution......................	37	1000
" " containing glucose..	48	1000
" " " "	27	5

the blood was treated similarly except that only the supernatant liquid was removed.

1 ml. of each suspension plus 0.05 mg. of neutralized nicotinic acid in 0.1 ml. of water and, as a control, 1 ml. of each suspension plus 0.1 ml. of its suspending medium were incubated 19 hours at 33–35°. The time required from venepuncture to the beginning of the incubation was 3 hours. After incubation the factor V assay of each mixture was made, and the percentage increase in each mixture containing nicotinic acid over its control was calculated.

The increase was 63, 64, and 65 per cent for the original blood, the cell suspension from which most of the leucocytes were removed, and the washed cells respectively. There were 700, 10, and 535 leucocytes per million erythrocytes in the preparations in the order given.

This experiment indicates that the synthesis of factor V was not a function of the number of leucocytes. It must be concluded, therfore, that human erythrocytes can synthesize factor V from nicotinic acid *in vitro.*

Comparison of the experiments shows that the difference in the extent of synthesis in serum and in Ringer-phosphate solution containing glucose obtained in the first experiment was due to the excessive handling of the cells.

However, even when the separation of the two types of cells was made under conditions unfavorable for synthesis, *i.e.* suspension in saline and excessive handling, synthesis of factor V by the erythrocytes to the extent of a 15 to 25 per cent increase could always be obtained. Such obvious damage as hemolysis or the presence of an inhibitor, *e.g.* 1 to 2 mg. of potassium oxalate per ml. of suspension medium, inhibits the synthesis. The oxalate inhibition occurs even with fresh untreated blood.

On the basis of the above experiments it may be suggested that the inability of Vilter, Vilter, and Spies (3) to demonstrate an increase in factor V when "carefully washed" erythrocytes were incubated with nicotinic acid was due to two factors. First, the the cells were damaged. Secondly, the relatively small synthesis performed by these cells could not be detected by the published method of Vilter, Vilter, and Spies (6) which is based upon the visual comparison of the bacterial growth elicited by dilutions of blood of 1, 1.5, 3, 6, and 12 ml. per 12,000 ml.

SUMMARY

Human erythrocytes can synthesize factor V from nicotinic acid *in vitro.* The presence of oxalate, the absence of glucose, suspension of the cells in sodium chloride solution, and excessive handling of the cells tend to diminish the synthesis.

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A SEASONAL STUDY OF THE IODINE CONTENT
OF THE BLOOD OF BIRDS*

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For some time investigations concerned with seasonal variations
in water metabolism have been carried out in this laboratory. In
one of these, Boyd and Johnston (1) reported a marked seasonal
change in the water content of the respiratory tract of normal
rats. To observe whether the changes found were peculiar to
rats, or whether they were of a more general significance, in 1939–
40 the investigation was extended to pigeons and poultry. This
afforded an opportunity to study at different seasons the iodine
content of the blood of birds, a subject upon which very little
work has been done.

Most investigators have reported more or less marked seasonal
cyclic changes in the thyroid gland. Seidell and Fenger (8) and
Fenger (3) found that the total iodine content of the thyroids of
sheep, pigs, and oxen was highest in the autumn and lowest in
the spring. Kendall and Simonsen (4) confirmed this observa-
tion and found, in addition, that the acid-insoluble iodine content
of the thyroid varied directly as the total.

While evidence of a seasonal change in the iodine content of the
thyroid has been consistent, evidence of a parallel change in the
iodine content of blood remains contradictory. Elmer (2) reviews
relevant data, largely written in German, published before 1938;
from his data it may be seen that earlier investigators observed a
low level in autumn and winter and a higher level in early spring
and summer, while later investigators denied the existence of any

significant seasonal change in the iodine content of blood. Perkin and Lahey, in 1940 (7), review conflicting evidence, largely written in English and French, and at the same time report no seasonal variation in blood iodine values obtained at various periods of the year from 745 normal human subjects. McClendon and Olson, in 1940 (5), reported no seasonal variation in the basal metabolic rate which frequently varies with the blood iodine.

The present investigation was carried out on animals which have not been investigated heretofore in this regard, and whose normal blood iodine level has not been established. The only previous report of the normal iodine content of the blood of birds is that of Stimmel, McCullagh, and Picha (9) who found that blood samples of three chicks contained 6, 8, and 10 γ per 100 ml. of blood respectively.

Method

For a period of 1 year, at approximately monthly intervals, ten pigeons and ten chickens were obtained from dealers, housed, and fed for 1 week in the laboratory and then killed without anesthesia by severing the cervical spinal cord. Blood was obtained by cutting quickly an available artery in the neck or wing and allowing the blood to flow, without coming into contact with the skin, into a vessel containing potassium oxalate as an anticoagulant. From 7 to 15 ml. of blood could be obtained in this manner.

The iodine content of samples of 10 ml. of blood was determined after Perkin's modification of von Fellenberg's titrimetric method (6). In cases in which 10 ml. of blood were not available, 5 ml. from one bird were added to 5 ml. from another bird of the same batch so that the iodine content observed would be a mean of the iodine contents of the two constituent samples. In all, 110 pigeons and 80 chickens were used.

Results

In Table I are tabulated the dates upon which samples of blood were taken for analysis from pigeons and from chickens, their respective mean iodine contents in γ per 100 ml., and the minimal and maximal values included in each mean. The values in Table I show that, throughout the year, the mean iodine content of the blood of birds remained relatively constant. The maximum

difference between any two means was 3.3 γ per 100 ml. for pigeons
and 3.1 γ per 100 ml. for chickens.   This difference could not be
significantly related to season.   Occasionally a mean difference
as great as the maximal occurred in two successive samplings
made within 2 or 3 weeks of each other.   For example, such in-
stances were observed on May 13 and 29 with pigeons and Sep-
tember 13 and 29 with chickens (see Table I).   In these specific

TABLE I

Iodine Content of Blood of Pigeons and Chickens Taken at Different Seasons of Year

Each mean represents analyses of blood samples from a total of ten birds.

	Date	Mean	Minimum variate	Maximum variate
		γ per 100 ml.	γ per 100 ml.	γ per 100 ml.
Pigeons	Aug. 14, 1939	5.3	5.2	5.5
	Sept. 13, 1939	4.0	3.7	4.4
	" 28, 1939	3.5	3.1	3.7
	Oct. 20, 1939	2.4	2.0	2.5
	Jan. 1, 1940	5.6	4.6	6.7
	" 28, 1940	3.4	2.1	7.9
	Feb. 29, 1940	3.2	2.2	5.8
	Mar. 28, 1940	4.7	3.1	6.2
	May 13, 1940	5.5	3.6	8.9
	" 29, 1940	2.3	2.0	2.8
	June 21, 1940	2.3	1.1	3.2
Chickens	Aug. 15, 1939	5.5	4.4	6.1
	Sept. 13, 1939	2.9	2.4	3.2
	" 29, 1939	4.1	3.0	5.3
	Jan. 28, 1940	5.6	2.7	9.1
	Feb. 29, 1940	2.5	2.2	2.8
	Apr. 9, 1940	3.6	1.7	5.0
	May 23, 1940	3.9	2.6	6.2
	June 22, 1940	4.2	2.4	7.3

cases, season certainly could not have accounted for the difference
between the means.   Rather one would suggest that the small
variations found between mean iodine values might be due to
individual variations.   The curve presented by Elmer ((2) Fig. 3)
shows, at first sight, a seasonal rise and fall but the difference
between the highest and lowest values is only 5.1 γ per 100 ml.;
most values are higher than the now usually accepted normal

limit for man and there is no statistical proof offered of a significant difference.

Statistical analysis of the data presented in this report failed to indicate any relation between season and the iodine content of the blood samples. Since too small a number of animals was sacrificed each month to permit of statistical analysis in monthly groups, the blood iodines of animals killed in 3 succeeding months were grouped together in all possible combinations (January, February, March; February, March, April; etc.) and compared in frequency polygons. In no case was there any indication that one 3 month group of iodine values differed significantly from any other 3 month group. When iodine values from November until April, inclusive, were compared statistically with those obtained from May until October, inclusive, no significant difference between the two seasonal groups with either pigeons or chickens was found.

These results prove that there is no gross seasonal variation in the blood iodine of birds. They do not prove that no *small* seasonal variation occurs. If such small variations do occur seasonally, to demonstrate them conclusively much larger numbers of animals kept under more uniform conditions throughout the year would need to be studied in order to reduce variation between individuals to a minimum.

Since the data were not related to season, they may be considered as a more or less uniform group of normal values. As such they may be analyzed to provide figures for normal variation of the iodine in the blood of pigeons and chickens. The mean iodine content of the blood of 110 pigeons was calculated to be 4.2 γ per 100 ml. with a standard deviation of 1.7, and with minimal and maximal variates of 1.1 and 8.9 γ. The mean iodine content of the blood of 80 chickens was calculated to be 4.1 γ per 100 ml., with a standard deviation of 1.6, and with minimal and maximal variates of 1.7 and 9.1 γ. These means are lower than the mean of 8.0 per 100 ml. reported by Stimmel, McCullagh, and Picha (9) for chicks, but they cover a great many more birds. They are somewhat lower also than the mean value of 6.8 γ per 100 gm. of human blood reported by Perkin and Lahey (7) from Boston, and than the mean value of 7.1 γ per 100 ml. found by the author from analyses of samples of blood from twenty-six normally active human subjects in this district.

SUMMARY

At approximately monthly intervals for a period of 1 year, ten pigeons and ten chickens were killed and their blood analyzed for its iodine content after the method of Perkin (6).

No significant seasonal variation in the iodine content of the blood samples was found.

The mean iodine content of the blood of 110 pigeons was 4.2 with a standard deviation of 1.7 γ per 100 ml., and of 80 chickens was 4.1 with a standard deviation of 1.6. These values were somewhat lower than an average of 7.1 γ per 100 ml. found in twenty-six normal human subjects by the same method.

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THE EFFECTS OF HIGH PRESSURE ON THE ACTIVITY OF PEPSIN AND RENNIN

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The general effects of high hydrostatic pressure on physical and chemical systems have been studied intensively by Bridgman (1) and by Cohen (2) over a pressure range of several thousand atmospheres. Considerable work has been done on the various effects of high pressure on biological systems. Bridgman (3) and Bridgman and Conant (4) studied denaturation by pressure of egg albumin and solutions of carboxyhemoglobin. Basset and his associates (5–9) reported the effect of high pressure on the activities of trypsin, diastase, sucrase, pancreatic lipase, and trypsinogen, as well as the use of pressure to control the toxicity of various sera, toxins, and venoms. Other investigations by Frankel and Meldolesi (10), Hite, Giddings, and Weakley (11), Larson, Hartzell, and Diehl (12), and Dow and Matthews (13) reported the behavior of enzymes, bacteria, and bovine blood, respectively, under similar conditions.

In this paper we are reporting on the effect of high pressure on the activity of pepsin and rennin. In the case of the former, crystalline pepsin was used. Since this is known to be a protein, the work with this enzyme should have double significance. Besides showing the effect of pressure on a typical enzyme, it should also throw some light on the denaturation of a pure protein as measured by an important biological property.

Methods

The method of Northrop (14) was used for the preparation of crystalline pepsin, and that of Anson and Mirsky (15) for the

measure of its activity. The peptic unit of these authors has been used as the unit of activity for the results computed from the data of this paper. The rennin solution was prepared by dissolving 100 mg. of a dry enzyme preparation (No. 259, The Wilson Laboratories) in 100 cc. of distilled water with a small crystal of thymol for a preservative. The substrate was prepared from milk powder to insure consistent and reproducible coagulation times. 50 gm. of whole milk powder (Klim, The Borden Company) was dissolved in 500 cc. of distilled water and 5 cc. of an 8 per cent solution of $CaCl_2$ added. 200 cc. of boiling water were further added to the mixture and, after cooling, 1 volume of 0.02 M sodium acetate-acetic acid buffer solution (pH 6.2) was added to 3 volumes of the milk. The method used for determining activity was a slight modification of those of Tauber and Kleiner (16) and Stone and Alsberg (17). A standard test solution of rennin was considered as one that clotted a 10 cc. sample of the milk in 10 minutes at 40°. Both enzyme solutions were freshly prepared prior to each pressure treatment.

The method of generating high hydrostatic pressure is that of Bridgman (1). Pressures were measured in the test chamber by observing the change of electrical resistance with pressure of a coil of manganin wire, previous work having shown that the change of resistance varies linearly with change of pressure.

The pressure treatments of the pepsin solutions were applied at a constant temperature of 35.5° by means of a thermostatically controlled water bath that surrounded the test chamber, but the treatments for the rennin solutions were performed at room temperature (23°) without precautions to keep a constant temperature. The activity of the first is specified (15) at the above temperature but that of the second is not supposed to be critically dependent on temperature over a moderate range.

10 cc. samples of the solutions were prepared for the pressure treatment by being placed in paraffin-lined containers that separated the samples from the surrounding liquid which transmitted the pressure in the test chamber. One type of container, a flexible tooth paste tube, proved to be very effective in providing mechanical separation of the liquids, although it was found that aseptic conditions could be maintained and separation obtained equally well by placing the samples in cylindrical tubes and cover-

ing with a thick layer of sterilized paraffin oil. Basset (5) and Hite *et al.* (11) stated that no effects were found in their results that were due to the nature of the containers they used, and the writers have obtained reproducible data by using both types of the containers that have been described.

Pressure was always applied isothermally, at such a rate that 5 or 10 minutes were required to generate a few thousand atmospheres of pressure. Accordingly, the maximum change of temperature due to generation or release of pressure can be considered to be a fraction of a degree.

Results

The principal experimental results are shown by Figs. 1 to 5. Peptic and rennin activities have been measured as functions of pressure and exposure time, at constant concentrations and temperatures. It appears from the nature of the curves that a simple interpretation is impossible on the basis of these data alone. However, it seems desirable to call attention to some of the significant characteristics of the curves.

Bridgman and Conant (4) found the denaturation of carboxyhemoglobin by pressure to be a first order reaction dependent on the pH of the solution. It is evident from the data presented here (Figs. 1, 2, and 3), at constant pressure and temperature, that the reaction rate is not of the first order. Moreover, as regards the data for peptic activity we have found in come cases that the activity depended on the buffer and its pH. The pH of the standard solution was 4.8. When a potassium chloride-hydrochloric acid buffer of pH 2.2 was used, no change of activity from that of the standard unbuffered solution over a pressure range of 6000 kilos per sq.cm. was found. But with a potassium-hydrogen phthalate buffer of pH 4.2, a decrease of 45 per cent from that of the unbuffered solution was measured. Rennin activity over a range of 1000 kilos per sq.cm. appeared to change in a buffered sodium acetate-acetic acid solution (pH 6.2) by the same amount as in the unbuffered solution of pH 5.5.

Peptic activity was practically unaffected by pressures of 1000 kilos per sq.cm. or lower, over an exposure time interval of 6 hours. The activities of both enzyme solutions under pressure were found to be functions of the temperature. A detailed study of the tem-
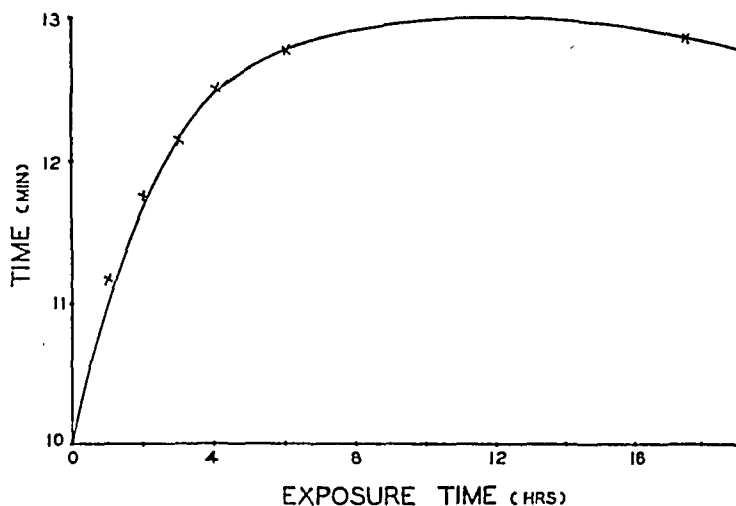
FIG. 1. Clotting time of rennin *versus* exposure time at 1000 kilos per sq.cm. The clotting time of the control was 10 minutes in every case.
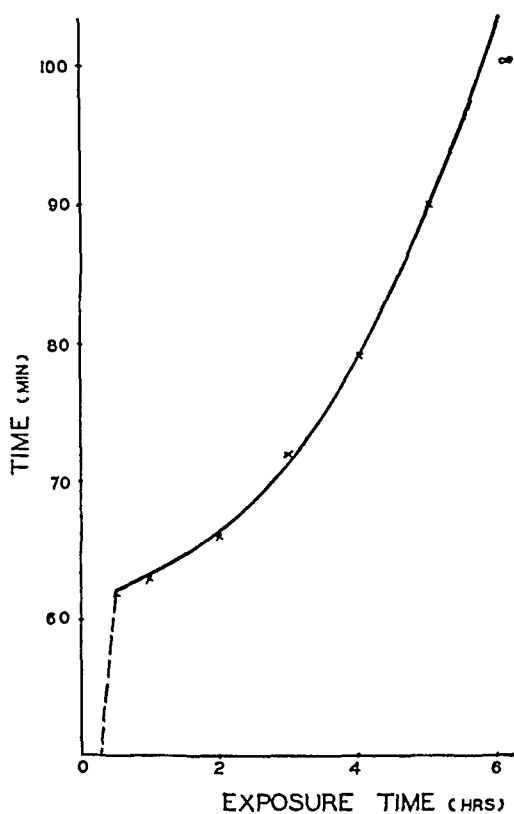
FIG. 2. Clotting time of rennin *versus* exposure time at 5000 kilos per sq.cm. The clotting time of the control was 10 minutes in every case.

perature effect was not made. For a pressure exposure of 5000 kilos per sq.cm. for 5 hours, the decrease of peptic activity was 25 per cent at 0° against 60 per cent at 35.5°. For rennin under similar conditions of pressure treatment, it was found that at 0° the coagulation time increased by 550 per cent, but at 23° the increase was 800 per cent, the activities varying inversely with the coagulation times.

The complicated variation of the activity *versus* exposure time curves at constant pressure suggests the existence of more than one reaction. In Fig. 3, for pepsin, particularly as regards the
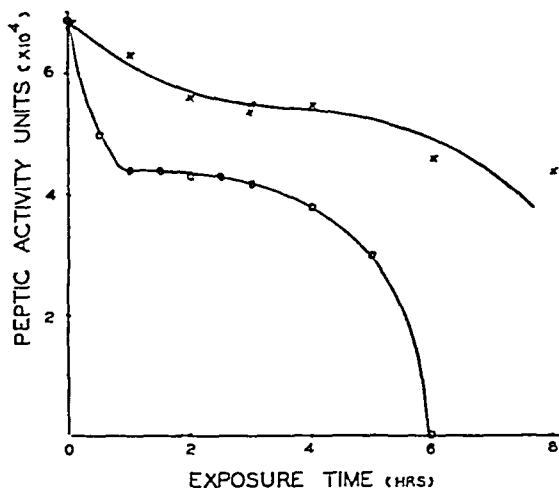
FIG. 3. Peptic activity *versus* exposure time at constant pressure. The upper curve is at 2500 kilos per sq.cm. and the lower at 4500 kilos per sq.cm.

curve at 4500 kilos per sq.cm., it is to be noticed that the slopes of the curves changed in sign as the exposure time interval increased. Figs. 1 and 2 for rennin show strikingly the effect of pressure on the clotting time. It is evident that the activity of rennin as a function of time at 1000 kilos per sq.cm. is quite different from that at 5000 kilos per sq.cm. Accordingly, for both enzymes, it appears to us that several reactions probably with quite different rates occurred during the exposure interval, and that these rates are influenced strongly by the magnitude of the pressure.

The activity *versus* pressure curves at constant exposure times did not vary considerably in nature from one curve to another. The curve of Fig. 4 for 1 hour's exposure time was found to be similar to the one for an exposure time of 4 hours. On the basis of these results it can be said that peptic activity was relatively uninfluenced by the length of the exposure time interval. Fig. 5 for rennin was obtained at a constant exposure time of 1 hour. Qualitatively, the activity curve (inverse of the clotting time
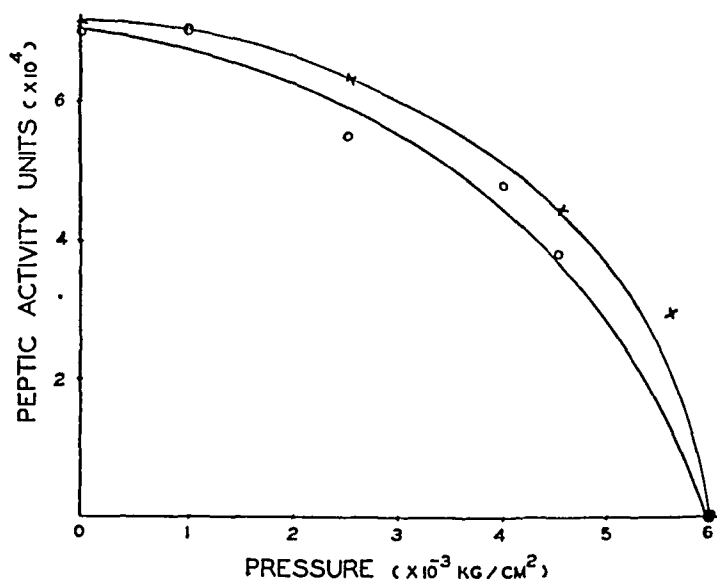
FIG. 4. Peptic activity *versus* pressure at constant exposure time. The upper curve is at 1 hour and the lower at 4 hours.

curve) was found to be similar in general nature to those of pepsin under like conditions.

Several experiments were repeated in order to observe the reproducibility of data.   There was no appreciable error due to control solutions of both pepsin and rennin, as in practically every case fresh solutions gave the same measured activities.   Under the pressure conditions there was greater possibility of error, although the error in the measurement of pressure alone according to standard methods was considered to be negligible.   As a measure of reproducibility, the peptic activity curve at 2500 kilos per sq.cm. of Fig. 3 has two values for an exposure time of 3 hours.

It will be seen that the difference is about 0.2 of a peptic unit, a value that is commensurate with the reproducibility obtained in general. However, it must be emphasized that the intrinsic errors in the reactions were not subject to control and it is very
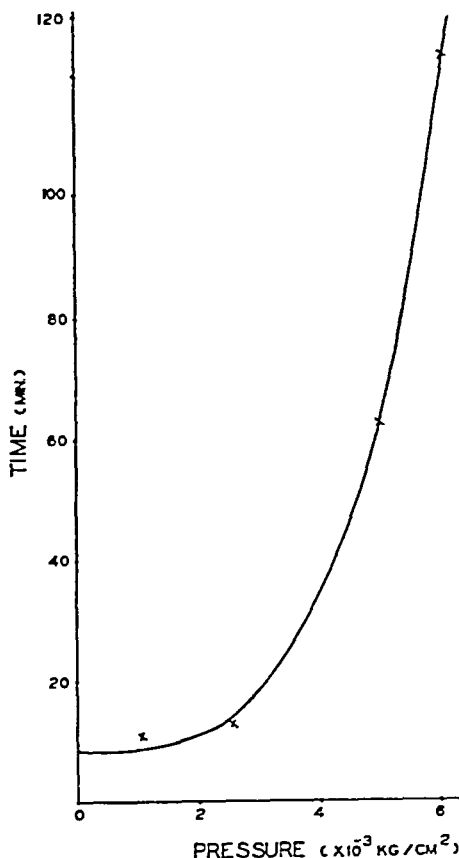
Fig. 5. Clotting time of rennin *versus* pressure at exposure times of 1 hour. The control clotted at 10 minutes in every case.

likely that these errors were responsible for the deviations observed on several of the curves, namely those of Fig. 4.

Hydrolysis under pressure might be expected to cause some of the complications that were observed. Amino nitrogen values

(Van Slyke) were measured for five samples of pepsin solution that had been treated with a pressure of 10,000 kilos per sq.cm. for 1 hour at 23°. No change of amino nitrogen content was found and it must be concluded that no appreciable hydrolysis resulted during the pressure treatment.

Although it is evident that these data show that the loss of activity of pepsin and rennin due to pressure is not a result of simple denaturation, the physical appearance of the denatured substance often was similar to that which had been denatured by heat. Both enzyme solutions were heavily coagulated by pressure treatment at 10,000 kilos per sq.cm., a result that appeared to be the same as when the solutions were heated to 100°.

Physically, denaturation by pressure is not similar to denaturation by heat. When a substance is heated at atmospheric pressure, energy is put into it and measured by the temperature rise. But when a substance is compressed isothermally, not only is work done externally but also internally by means of the attractive forces of the molecules. For example, in isothermal compression of the solutions used in this study to a pressure of 1000 kilos per sq.cm. it can be shown by thermodynamics that 7 times more energy flows out of the solutions than is put in by the work of compression. Energy, then, cannot be the sole factor for denaturation by pressure.

SUMMARY

The loss of activity of pepsin and rennin has been studied as a function of pressure and exposure time. Activity decreased with pressure increase at constant exposure time and was completely destroyed at pressures ranging between 5000 and 6000 kilos per sq.cm. Activity decreased also with increase of exposure time at constant pressure, but to a lesser degree. Pressure was important in determining the activity of these enzymes, but time of exposure was relatively unimportant. Loss of activity appeared to be dependent on certain buffers and the pH. A few observations showed that inactivation of pepsin and rennin by pressure depended strongly on the temperature.

It has been shown that denaturation by pressure was the probable cause for inactivation, although the reaction is not a simple monomolecular one. Pressure treatments as high as 10,000 kilos

per sq.cm. caused no change in amino nitrogen content and it has been concluded that no hydrolysis was caused by pressure. The product of denaturation by pressure appeared similar to that which had been denatured by heat. It has been shown that the energy relations must be quite different in the two processes. Consequently, it can be said that energy changes are not alone responsible for denaturation and deactivation of the enzymes pepsin and rennin by pressure.

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THE BIURET REACTION IN THE DETERMINATION OF SERUM PROTEINS

I. A STUDY OF THE CONDITIONS NECESSARY FOR THE PRODUCTION OF A STABLE COLOR WHICH BEARS A QUANTITATIVE RELATIONSHIP TO THE PROTEIN CONCENTRATION*

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The work of Autenrieth (1, 2) showed that the biuret reaction could be employed as the basis of a quantitative colorimetric method for the determination of albumin and globulin in blood serum, urine, and ascitic fluid. The method was not widely adopted because there was difficulty in securing a suitable standard protein solution that would not deteriorate in a very short time. Hiller (3) used the method of Autenrieth in the development of her procedure for the determination of albumin and globulin in urine. As a standard she used a commercial preparation of biuret (Kahlbaum reagent (4)). A pure dry sample of biuret, suitable for weighing, is not easily made, and in a short time we were unable to obtain this reagent from any supply house. In 1935 Fine (5) published a procedure in which he made a standard protein solution by diluting blood serum with a solution of 0.9 per cent sodium chloride until the protein concentration was approximately 0.24 per cent. The protein concentration was obtained from a nitrogen determination by the Kjeldahl method. This standard could be kept for at least several months in the presence of chloroform.

For several years we have successfully used protein standards

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prepared from diluted sera in our determination of serum proteins with the biuret reaction.   Diluted rabbit sera were much better than dog or human sera because the stock solutions remained clear for a much longer time.   Rabbit serum standard solutions have been kept for periods of over 6 months when preserved with a crystal of thymol, and as long as they remained clear the protein nitrogen values by the Kjeldahl method were always within the experimental error of the values obtained immediately after the solutions were made.   The biuret color solutions were prepared as recommended by Hiller (3); that is, the final concentration of sodium hydroxide was 3 per cent, and the amount of 20 per cent copper sulfate added for a final volume of 10 cc. was 0.25 cc.   The separation of the globulin and albumin was accomplished at 25° (Robinson, Price, and Hogden (6)) by the method of Howe (7), with the modifications of filtration technique suggested by Robinson, Price, and Hogden (8).

The biuret reaction for the estimation of serum proteins has certain advantages over other colorimetric procedures.   The color intensities developed by the Folin phenol reagent are different for albumin and globulin (Greenberg and Mirolubova (9), Minot and Keller (10)).   Autenrieth (2) noted that serum albumin and serum globulin have the same biuret color value per gm. of protein. Fine's results (5) suggested that there might be a small difference. When a globulin standard was used to estimate albumin, the results tended to be low, and when an albumin standard was used to estimate globulin the results tended to be high.   He concluded that globulin might have a biuret color value slightly higher than that of albumin.   The differences were practically within the error of measurements, so that he recommended the use of the diluted serum standard for the determination of serum albumin. In this respect the work of Lieben and Jesserer (11) is important. From their studies of the biuret reaction they conclude that under the proper conditions the color intensity and color tone of the biuret mixtures of a variety of proteins and protein derivatives are equal at equal weight concentrations of the solutions, and independent of the molecular size and amino acid content.   On the other hand the work of Sizer (12), who has determined the transmission spectra of biuret color solutions with the Hardy recording photoelectric spectrophotometer, indicates that the

actual per cent transmission at a given wave-length and also the shape of the curve vary with the nature and concentration of the respective proteins. Results obtained in this laboratory show that the optical density at 560 mμ of biuret color solutions of protein is essentially the same per unit weight of protein for total serum protein, serum albumin, and protein recovered from pathological urine and ascitic fluid. Likewise the optical density values at this wave-length are the same for equal concentrations of protein from the blood sera of humans, dogs, and rabbits. With methods in which the Folin phenol reagent is used the color factors for human serum proteins are slightly different than those for dog proteins. Greenberg (13) found that the color developed by the phenol reagent is dependent on the age of the serum, as the color obtained decreases with time as the serum stands. We have never observed such a decrease in color with the biuret reaction. Moreover the stability of color obtained in the various procedures with molybdate-tungstate reagent has never approached that obtained with the biuret reaction.

After using the biuret reaction for several years, we were not convinced that we were producing the color in the best environment. Therefore, in this investigation, we obtained information on the stability of the biuret reaction color, the reproducibility of this color for any given protein concentration, the best concentration of alkali, and the most ideal amount and concentration of copper sulfate to be added. In a system where soluble protein is present with a precipitate, there is always a danger that some of the protein might be lost from the solution. When the biuret color is developed at the same time in an unknown solution and in a protein standard solution of approximately the same protein content, equal amounts of protein may be lost from both solutions, and in the color comparison no error would be introduced. With the application of photoelectric photometry and spectrophotometry for the measurement of color intensities, it becomes feasible and indeed very convenient to dismiss the making of a standard color with each determination. However, with such methods, it is important that the conditions under which the color is developed for any individual sample are the same as those used for the photometric standard curves that might have been obtained months or years earlier. In order to detect any loss of protein

we determined the protein nitrogen directly on the biuret color solutions and compared the values with those obtained on dilutions of the protein solutions to which no copper had been added.

Methods

Measurement of Color Intensities—A study of the absorption spectra of the biuret color solutions of serum proteins reveals a
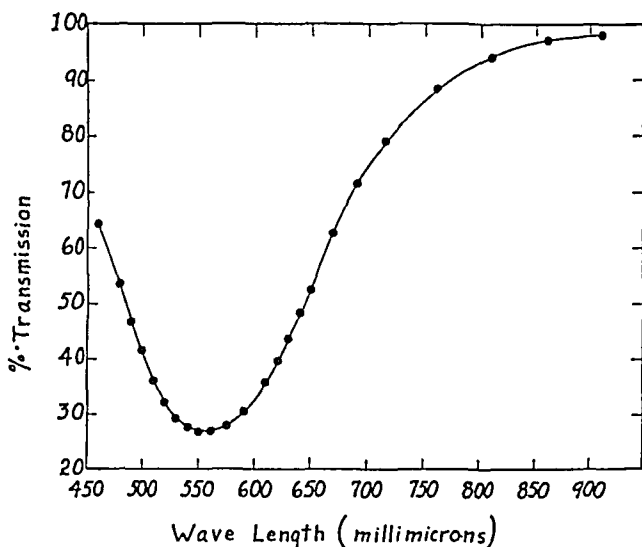
Fig. 1. Transmission curve of biuret color solution. To obtain these values a biuret color solution was used which had been prepared from rabbit serum protein. The measurements were made on the Coleman double monochromator spectrophotometer Model 10S. As this instrument gave maximum values that were 10 mµ lower than those obtained with the Bausch and Lomb universal spectrophotometer (used in all subsequent work), the readings for the Coleman instrument have been labeled to correspond to the scale of the Bausch and Lomb instrument which had been checked with the two sodium lines at 589 and 589.6 mµ.

broad absorption band between wave-lengths 550 and 560 mµ. The nature of a transmission curve is shown in Fig. 1.   The curve is not only similar in shape to that reported by Sizer (12) but the position of maximum absorption also agrees with that found by him for egg albumin and edestin solutions when he used the biuret reaction as performed by Lieben and Jesserer (11).   The region of maximum absorption is best suited for quantitative studies of the biuret color-protein relationship.

It is essential for good photometric measurements that the solutions approach optical clearness. Therefore, it was necessary to determine the conditions that would give solutions meeting this requirement. Early in this work we concluded that clearer solutions could be obtained by dissolving serum proteins precipitated with trichloroacetic acid in alkali than by using blood serum directly. In the latter case the color solutions have a definite opalescence, and measurements of optical densities show that the color intensities are changing during periods from $\frac{1}{2}$ to 3 hours after color development.

For the accurate measurement of color intensity we have determined the optical density at wave-length 560 mμ with the Bausch and Lomb universal spectrophotometer. The collimator slit was set at 1.0 on the scale of the instrument, which gives a purity of spectrum that is 1.4 per cent less than the maximum possible. The ocular slit was set at a width of approximately 3.0 mμ. In this report practically all measurements were made with a depth of solution at 30 mm. Using the vertical attachment of the instrument which allows the liquid column to be adjusted to any depth up to 60 mm., we found the clear biuret color solutions obeyed Lambert's law, and with the changes in concentration the readings also followed Beer's law. In an attempt to obtain differences which might be caused by the presence of turbidity we also made readings at 700 mμ. In order to obtain sufficient illumination we were here forced to open the collimator slit to 2.0 on the scale, which reduces the purity of the spectrum to 5.7 per cent less than the maximum possible. However, as the work progressed, it was realized that there were other factors which influenced the latter measurement.

Preparation of Clear Biuret Color Solutions—A 1:25 dilution of blood serum was made with a 0.9 per cent solution of sodium chloride. For each 100 cc. of the biuret color solution required for the experiments, 50 cc. of the diluted serum were mixed with an approximately equal volume of a 10 per cent solution of trichloroacetic acid. In our procedure we usually placed 25 cc. of the diluted serum in a 50 cc. Pyrex centrifuge tube and added the trichloroacetic acid solution, stirring with a fine glass rod. The mixture was centrifuged until the protein precipitate was well packed and the supernatant liquid clear (about 15 minutes at

3600 R.P.M.).   The latter was poured off and the tube inverted on a filter paper and drained.   The protein precipitate was not washed, but was dissolved directly in a few cc. of 3 per cent sodium hydroxide.   This solution was facilitated by the incorporation of 1 or 2 drops of the alkali with the precipitate to form a smooth paste, and then a few cc. of the alkali were added to dissolve the protein completely.   It is important before proceeding further to examine the solution for small gel-like particles which often form when a protein is dissolved in alkali.   When these particles were present, the solution was stirred or allowed to stand until all of them had disappeared, for their presence invariably resulted in low color intensity values.   The dissolved protein from the 50 cc. of diluted serum was transferred quantitatively to a 100 cc. volumetric flask, the centrifuge tubes being washed several times with the 3 per cent sodium hydroxide solution.   After addition of alkali to bring the volume of the solution to within 5 cc. of the mark on the flask, 2.5 cc. of a 20 per cent solution of copper sulfate ($CuSO_4 \cdot 5H_2O$) were added.   The solution was made up to 100 cc. with the alkali and the mixture shaken vigorously for 1 minute in order to develop the purplish red color.   We usually transferred the mixture to a 125 cc. Erlenmeyer flask in order to obtain easy mixing with the insoluble cupric hydroxide.   After standing for 1 or 2 hours the mixture was centrifuged for a few minutes to remove the precipitated cupric hydroxide.   The supernatant liquid, which was carefully pipetted off, was in most cases fairly clear, but, after many experiments, we concluded that a more uniformly clear solution could be obtained when this supernatant solution was filtered through one sheet of 9 cm. Munktell No. 00 paper, always discarding the first portion passing through the paper.

Results

Stability of Biuret Color—Biuret color-cupric hydroxide mixtures were made up by the procedure outlined above and portions were filtered at intervals of $\frac{1}{2}$, 1, 2, 4, 24, and 48 hours.   No special precautions were taken to keep the solutions in the dark during the day, but they were kept in a refrigerator overnight.   It had been observed previously that the purplish red color tone of the biuret solutions appeared to change toward a red tone on standing

for 24 hours. However, the density values at 560 mμ measured at various time intervals up to 48 hours showed no appreciable change. On the other hand the density measurements at 700 mμ decreased in value. These observations are in agreement with those of Jesserer (14) who found that the measurements made with the blue filter were constant for long periods of time, but that those made with the red filter decreased in color value. We believe, as stated by Sizer (12), that in this two component system the optical density value at 560 mμ, which appears to be so stable, is a measure of the copper-protein complex, and the decrease in the value at 700 mμ is an indication of cupric hydroxide being removed from the system.

Protein Nitrogen in Biuret Color Solutions—The possibility that the biuret color solutions contained an amount of protein not comparable with that of the original serum concerned us in this study. Protein nitrogen was determined by the Kjeldahl digestion method on both saline dilutions and biuret color solutions from the same serum. The usual factor of 6.25 was used to convert the protein nitrogen to protein. In the case of the saline dilution the protein nitrogen was obtained by subtracting the non-protein nitrogen from the total nitrogen. There were eight albumin determinations, in which the albumin nitrogens were determined by the Kjeldahl procedure on sodium sulfate filtrates and also on biuret color solutions made from these filtrates. The results in Table I indicate that no protein was lost by centrifugation or by filtration under these conditions (3 per cent NaOH, 20 per cent $CuSO_4 \cdot 5H_2O$). On the other hand, if the cupric hydroxide precipitate is poured on the paper with the supernatant liquid, an appreciable lowering of the nitrogen content of the filtrate occurs owing perhaps to adsorption of protein by the heavy layer of precipitate on the paper. The optical density at 560 mμ was reduced proportionately to the amount of protein lost. We realize that in the determination of nitrogen from biuret color solutions there may be a small volume error owing to the fact that these samples were made up to a definite volume, part of which was occupied by the insoluble cupric hydroxide. These results indicate that the error from this source must be small, and, for the accuracy demanded of most serum protein determinations, it can be neglected.

Table I

Comparison of Serum Protein Values Determined on Saline Dilution of Serum and on Biuret Color Solutions Developed from Same Serum Proteins

The serum protein was calculated from nitrogen determinations.

	Date	Subject No.	Saline dilution of serum	Biuret color solutions	
				Centrifuged supernatant solution	Filtered solution
			gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
Dog	Nov. 2	3	5.9	5.9	
	" 2	4	6.0	5.9	
	" 3	8	5.7	5.9	
	" 3	105	6.6	6.7	
	" 14	105	6.5	6.6	6.6
	" 20	6	6.2	6.2	6.3
Rabbit	Oct. 30	1869	5.8	5.8	
	Nov. 8	1869	5.9	5.8	
	Oct. 30	117	5.8	5.6	
	" 30	1870	6.4	6.3	
	Nov. 14	1870	6.4	6.5	6.6
	" 17	308	6.4	6.5	6.4
	" 22	308	6.4	6.4	6.4
	Dec. 6	308	6.5	6.5	6.5
	Nov. 7	307	6.0	6.0	6.0
	" 20	307	6.0	6.1	6.1
	" 22	307	6.0	6.0	6.0
	Jan. 30	307	5.7	5.7	5.7
	Dec. 1	38	5.7		5.5
	" 7	38	5.7	5.7	5.7
	Apr. 24	38A*	4.5		4.4
	Dec. 8	306	5.6	5.6	5.6
	Feb. 7	210	6.0		6.0
	" 15	210	6.0	6.0	
	" 29	338	6.5	6.6	6.7
	Mar. 1	310	5.8		5.8
Human	Jan. 16	1	7.0	7.1	7.1
	" 24	2	6.7		7.0
	" 24	2A	4.4		4.3
	" 24	3	7.9		8.1
	" 24	3A	5.2		5.0
	" 24	4	7.1		7.3
	" 24	4A	5.0		4.9
	Feb. 9	.5	4.6		4.5
	" 24	6	4.9		4.8
	" 24	6A	2.7		2.6
	Mar. 6	7	8.0		8.1
	" 6	7A	3.0		2.8
	May 2	8A	5.1		5.0
	" 2	9A	5.1		5.0

* In the albumin determination, globulin was precipitated with 1.5 M sodium sulfate. The albumin nitrogen was determined directly on the filtrate.

Effect of Filtration on Color Intensity—Although the biuret color solutions show no loss of protein nitrogen on filtration, the process caused a lowering of the optical density at 560 and 700 mμ. A blue coloration of the filter paper was always observed after an apparently clear solution had been filtered through a dry sheet of paper. At first this decrease of densities, which was small, was thought to be only more of the cupric hydroxide coming out of solution. We considered the possibility that, if the solution were refiltered a number of times, all of the increment known as the blue component might be removed and the color of the solution that remained might be the color of the copper-protein complex.
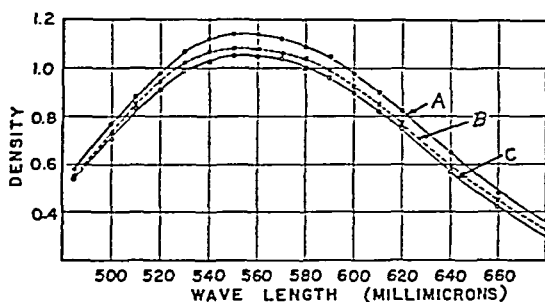
Fig. 2. Effect of refiltration on absorption curve of biuret color solutions. Curve A = filtered once through No. 00 Munktell paper; 18.14 mg. of nitrogen in 100 cc. of color solution. Curve B = refiltered twice through the same paper; 18.32 mg. of nitrogen in 100 cc. of color solution. Curve C = refiltered four times through the same paper; 18.26 mg. of nitrogen in 100 cc. of color solution.

Fig. 2 illustrates absorption curves of the biuret color developed from rabbit serum protein filtered once, and refiltered twice and four times through the same paper. Except for the decrease in the optical densities, there is no alteration of the absorption curve in the region 485 to 680 mμ. Analyses of the filtrates for nitrogen content indicated that no protein had been lost, but even after four refiltrations there was still a slight lowering of the density values. It was concluded that no constant density value was obtainable by refiltration, and that the decrease in optical density ·was greater than could be accounted for by the excess free cupric hydroxide alone. The only explanation which can be given for

this phenomenon is that the copper-protein complex must be very unstable, and the electric charges on the paper disturb this equilibrium.   These experiments made us realize that the filtered color perhaps did not represent an absolute color value for a copper-protein compound.   When the solution is filtered once through Munktell paper, the color loss is very small.   Over a wide range of protein concentrations a color value of the filtered sample gives a direct proportionality with the protein nitrogen.

We would like to emphasize that the values obtained with the use of different types of filter paper are not always comparable. For example, the same size sheet of Whatman No. 50 paper removed decidedly more color than did the Munktell No. 00 paper. A linear relationship between the optical density at 560 mμ and the protein nitrogen of the biuret color solution was also obtained after the solution was filtered through the Whatman paper; however, the ratio of density value to the nitrogen value was lower than that for the Munktell paper.

Amount of 20 Per cent Copper Sulfate Added—Lieben and Jesserer (11) found that the amount of 20 per cent copper sulfate could be varied without any change in the biuret color intensity if the protein concentration in 3 per cent sodium hydroxide were kept constant.   Considerable excess of copper sulfate evidently had no influence.   In their experiments the amount of copper sulfate never fell below 2 cc. per 100 cc. of solution.   We were interested in confirming this observation on a solution in which the protein nitrogen had been determined directly.   Three biuret color solutions were made up with the same amounts of diluted, alkaline, rabbit serum protein, but varying amounts of 20 per cent copper sulfate were added in the preparation of each mixture. The amounts of the copper salt introduced into 100 cc. of color solution were 1.25, 1.87, and 2.50 cc. respectively.   All solutions were centrifuged and the supernatant liquid filtered through Munktell paper.   The optical density of each sample was measured at intervals between 485 and 680 mμ.   The values shown in Table II indicate that, throughout the spectrum measured, all readings agreed within experimental error.   The nitrogen values of these color solutions, 18.1, 18.1, and 18.0 mg. per 100 cc., show that the varying amounts of copper had produced no significant change.   From these results it is evident that measurement of

the amount of copper sulfate solution with any degree of accuracy is unnecessary, as excess copper must be precipitated and does not contribute to the color value.

Effects Observed with Weak Solutions of Copper Sulfate—Since most of the copper added to alkaline solutions is immediately precipitated as cupric hydroxide, it is necessary after the intro-

TABLE II

Effect of Varying Amount of 20 Per Cent Copper Sulfate Added to Rabbit Serum Protein Dissolved in 3 Per Cent Sodium Hydroxide

All solutions were filtered through Munktell No. 00 paper.

20% $CuSO_4 \cdot 5H_2O$ per 100 cc., cc.	1.25	1.87	2.50
N, mg. per 100 cc.	18.1	18.1	18.0

Density values of biuret color solutions. Depth, 30 mm.			
Wave-length	Sample A	Sample B	Sample C
$m\mu$			
485	0.56	0.56	0.57
500	0.73	0.74	0.74
510	0.84	0.85	0.85
520	0.94	0.94	0.96
530	1.03	1.02	1.03
540	1.07	1.07	1.07
550	1.10	1.10	1.09
560	1.10	1.10	1.09
570	1.08	1.08	1.07
580	1.05	1.05	1.04
590	1.00	1.00	0.99
600	0.93	0.93	0.93
620	0.78	0.78	0.78
640	0.61	0.62	0.61
660	0.46	0.46	0.46
680	0.34	0.34	0.34

duction of small amounts of 20 per cent copper sulfate to shake the mixture vigorously in order to develop the biuret color. It occurred to us that it might be better to dissolve the protein in slightly stronger alkali and to add the same amount of copper in a more dilute solution. Therefore, 25 cc. of 2 per cent copper sulfate solution were used instead of 2.5 cc. of the 20 per cent solution. The final concentration of the sodium hydroxide was still main-

tained at 3 per cent.   With the dilute copper sulfate solution the color developed immediately with very little shaking.   The optical density at 560 mμ was close in most cases to that observed when 20 per cent copper sulfate was used, but the density values at 700 mμ were generally higher.   Analyses of the color solutions for protein nitrogen content showed low protein values, but after the mixtures had been standing in the presence of the precipitated copper for several days the nitrogen contents approached the correct values obtained on saline dilutions of serum.   The amount of cupric hydroxide which remained dissolved in the solution was always greater when dilute copper sulfate solutions were used, so that the ratio of optical density to the protein nitrogen value was higher than with the use of 20 per cent copper sulfate.   Moreover the variability of results encountered with the dilute copper sulfate introduced a factor of uncertainty never encountered with the 20 per cent copper sulfate.   When smaller amounts of the diluted copper sulfate were used, the protein loss was greatly reduced.   It is concluded that the precipitation of the copper in a much finer state of dispersion brings down some of the protein, and that in the course of time some of this protein is again redissolved.

Effects of Concentration of Sodium Hydroxide—The color of the biuret reaction is developed in the presence of alkali and destroyed when acid is added.   The stability of the protein-copper complex is somewhat dependent on the alkaline strength of the solution. However, as the concentration of alkali is increased, the blue color component, which is no doubt cupric hydroxide, forms a significant part of the total color.   When the copper salt (20 per cent copper sulfate) is introduced in 3 per cent sodium hydroxide and the solution filtered through Munktell No. 00 paper, the optical density at 560 mμ in a depth of solution of 30 mm. is only 0.02, a value which approaches the experimental error obtained for the protein solutions.   When the copper salt is added to 10 per cent sodium hydroxide and put through the same procedure, the density value is 0.45.   If this increment of color is also present when the biuret color is developed in a protein solution, it would be an appreciable proportion of the total color of the solution.   It is known that such an influence is present because the optical density-protein nitrogen relationships indicate that there is a considerably greater

absorption with the stronger alkaline mixtures than with the weak ones. If the alkali concentration is reduced below 3 per cent, there is a tendency for the protein nitrogen values to be low. Table III shows an experiment where this is evident. The trichloroacetic acid-protein precipitate from rabbit serum was dissolved in 3 per cent sodium hydroxide. A nitrogen determination was made on a 1:1 dilution of this stock solution (control Sample A). Samples B to G inclusive also are 1:1 dilutions of the stock solution. For each sample the proper amounts of copper salts, alkali, and water were added to give the final concentrations listed in Table III. Solutions D and F which were prepared with a

TABLE III

Effect of Varying Concentration of Sodium Hydroxide and Concentration of Copper Sulfate Solution Added on Protein Nitrogen Values of Biuret Color Solution

Sample	Final concentration of NaOH	Amount of 2 per cent $CuSO_4 \cdot 5H_2O$ added for 100 cc. solution	Amount of 20 per cent $CuSO_4 \cdot 5H_2O$ added for 100 cc. solution	Determined N (Kjeldahl) in color solution	Loss of N	Calculated protein concentration of original serum
	per cent	*cc.*	*cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*gm. per 100 cc.*
A	3.0			23.10		7.22
B	1.5		2.5	21.70	−1.40	6.78
C	1.5	25.0		21.26	−1.84	6.65
D	3.0		2.5	23.10	0.00	7.22
E	3.0	25.0		21.86	−1.24	6.83
F	6.0		2.5	23.20	+0.10	7.25
G	6.0	25.0		22.51	−0.59	7.04

20 per cent solution of copper sulfate gave nitrogen values in agreement with that of the control Solution A. Therefore the alkalinity of the solution may be increased without altering the protein value. On the other hand Solution B, in which the concentration of alkali was reduced to 1.5 per cent, gave a low nitrogen value, indicating that under these conditions protein was removed with the excess copper. Dilute copper sulfate was used in Solutions C, E, and G, and in all three cases the protein nitrogen content was low, the magnitude of the loss varying inversely with the concentration of alkali.

Addition of Reagents Directly to Diluted Alkaline Serum—In the

method introduced by Kingsley (15) the ·final concentration of alkali is approximately 8.7 per cent, and the amount of copper sulfate introduced is small so that no excess cupric hydroxide is precipitated. Also, Kingsley adds the serum directly, which eliminates precipitation of the protein. We have made determinations according to the Kingsley method, analyzing for protein nitrogen in the color solution. The readings in each case were made exactly 25 minutes after color development, but the solutions were not what we would call clear. In Fig. 3 is shown the
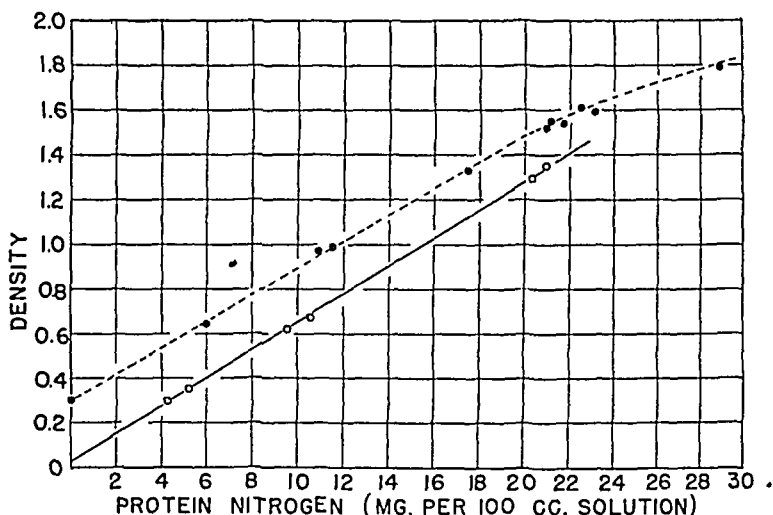
FIG. 3. Relation of the density value at 560 mμ to the protein nitrogen content of the solution. Depth of solution = 30 mm. The copper sulfate solutions were added directly to serum-NaOH solutions. ● represents 8.7 per cent sodium hydroxide, 10.9 cc. of 1 per cent $CuSO_4 \cdot 5H_2O$ per 100 cc., color read directly; O 3 per cent sodium hydroxide, 2.5 cc. of 20 per cent $CuSO_4 \cdot 5H_2O$ per 100 cc., color read after centrifugation and filtration.

relationship between the optical density and protein nitrogen on a series of determinations by this procedure. The point indicated at 0 concentration of protein nitrogen on the ordinate is the value obtained with the concentrations of copper and sodium hydroxide employed by Kingsley; that is, 0.5 cc. of 1 per cent copper sulfate solution was added to 4.0 cc. of a 10 per cent sodium hydroxide solution. It should be noticed that the line is not straight, but curves toward the horizontal at higher concentrations of protein nitrogen. This may be due, in part, to the fact that the amount

of free cupric hydroxide in solution decreases as the protein nitrogen increases, and with high concentrations of protein nitrogen one also encounters the possibility that there may be insufficient copper to combine with the protein. Varying degrees of turbidity introduce further factors of uncertainty.

Fig. 3 also shows the values obtained by adding 2 cc. of serum to 3 per cent sodium hydroxide and developing the color with 2.5 cc. of 20 per cent copper sulfate for 100 cc. of the solution. All solutions were centrifuged and the supernatant liquid filtered through Munktell paper. These points fall on a line which prac-
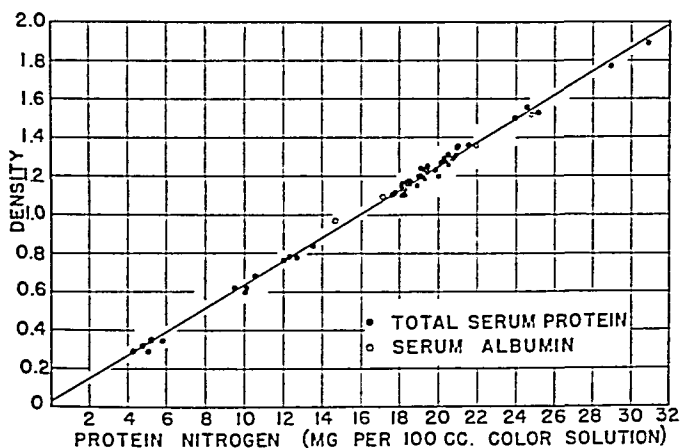
Fig. 4. Biuret reaction on rabbit serum protein. Relation of the density value at 560 mμ to the protein nitrogen content of the solution. Depth of solution, 30 mm.

tically coincides with the line in Fig. 4 for which the colors were developed in alkaline solutions of the precipitated proteins. However, the protein nitrogen determinations on the colored solutions in which the serum had been added directly to alkali were usually low when compared with determinations made on the diluted solutions to which no copper had been added. When these mixtures stood for a few days in the presence of the excess cupric hydroxide, the density values and the protein nitrogen increased proportionately. The fact that the observed density values are directly related to the nitrogen contents of the supernatant solutions would seem to indicate that there are no factors in these sera

which would seriously interfere with the biuret reaction when serum is added directly to alkali.   There is a danger of obtaining low protein nitrogen values when this amount of copper is added to a solution containing serum diluted in 3 per cent sodium hydroxide, owing to the precipitation of protein.

Data Obtained on Reproducible Color Solutions—From the foregoing experiments we were convinced that the most reproducible color solutions could be obtained by the addition of a 20 per cent solution of copper sulfate to a solution of the protein in approximately 3 per cent sodium hydroxide.   In order to obtain information on the quantitative applications of these color solutions with reference to the protein nitrogen content, a series of determinations was carried out on rabbit serum protein and rabbit serum albumin.   In each case the protein nitrogen was determined on the solution which had been read in the spectrophotometer.   On many of these samples the protein nitrogen was determined also on saline dilutions of the serum, and in every case the serum protein concentrations calculated from the nitrogen values of the two solutions were within experimental error.   In this group there are 55 determinations on sera obtained at various times from eleven rabbits.   The results shown in Fig. 4 indicate a linear relationship between the density value at 560 mμ and the protein nitrogen in the solution.   The line of these experimental points was calculated by the method of least squares.   The equation for this line is $D_{560} = 0.0615N + 0.02$, where D_{560} is the density at 560 mμ at a depth of 30 mm. and N is the mg. of nitrogen per 100 cc. of color solution.   The standard error of estimate for these density values is 0.0232; the standard error of the regression coefficient is 0.0005.   At 0 nitrogen concentration the line crosses the ordinate at the density value of 0.02, which is practically the density reading obtained by treating the alkaline mixture in the same manner as the biuret color solution.   This low value indicates that the soluble cupric hydroxide is contributing very little color to the solution, and therefore the density values are a direct measure of the copper-protein complex.

The density values at 700 mμ, which varied from 0.18 to 0.42, also indicate a linear relationship with the nitrogen determinations, although the relative experimental error is greater owing to the low density readings at this wave-length and to the fact that the human eye is less sensitive to color change at this portion of the

spectrum. The equation for the line calculated from the determinations by the method of least squares is $D_{700} = 0.0128N + 0.04$. The standard error of estimate is 0.0210 and the standard error of the regression coefficient is 0.0006.

From the experimentally determined line of Fig. 4, total serum proteins and albumins of rabbit sera could be determined from the optical densities with a fair degree of accuracy and in a much shorter period of time than was necessary for the Kjeldahl determination. As our greater interest was in human blood sera, it was important to find out whether the experimental points of a series from this species fell on the line established for rabbit sera.
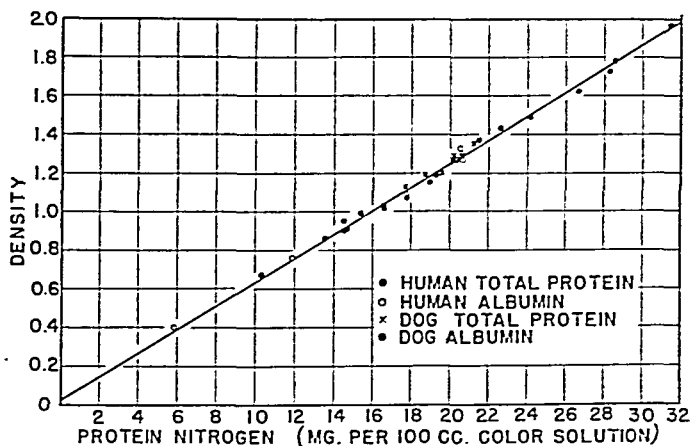
Fig. 5. Biuret reaction on human and dog serum. Relation of the density value at 560 mμ to the protein nitrogen content of the biuret color solution. Depth of solution, 30 mm.

A series of determinations by the same procedure used on rabbit sera was made on human and on dog serum proteins. The color readings were made under the same conditions, and the protein nitrogen values were determined directly on the biuret color solutions by the Kjeldahl method. In Fig. 5 we have plotted the experimentally determined points and show their relationship to the line established for rabbit sera. These points fall on both sides of this line and the values for dog and for human protein are intermingled in a manner which led us to the conviction that the biuret color values of the proteins from the sera of these species are the same.

If we calculate the equation of the best line for the values of thirty-three determinations on dog and human serum proteins, close agreement is found with the equation for the rabbit serum values.   The equation for this line is $D_{560} = 0.0606N + 0.04$. The standard error of estimate for these density values is 0.0188 and the standard error of the regression coefficient is 0.0006.

These determinations show that, with the proper conditions, the biuret reaction may be used as a sound quantitative procedure for the estimation of blood serum protein concentrations.   When clear biuret solutions are obtainable, we are convinced that this colorimetric method has such a reliability that Kjeldahl determinations are unnecessary.

In many instances sufficient serum may not be available for the filtration, and, therefore, determinations must be made directly on the supernatant solution after centrifugation.   When such determinations are made, a relationship similar to that shown in Fig. 5 (optical density at 560 mμ to mg. of nitrogen per 100 cc. of color solution) is obtained, except that the density values for a given protein concentration are slightly greater than those obtained when the samples are filtered.   This increase in color intensity is due to the presence of more cupric hydroxide in the solution and to the avoidance of the small loss observed after filtration.   Omitting the filtration process, we made forty-one determinations of the optical density and the protein nitrogen on the color solutions prepared from human, dog, and rabbit serum proteins.   The equation of the line calculated for these experimental points was $D_{560} = 0.0639N + 0.05$.   The standard error of estimate for these density values, 0.0346, was higher than that for the filtered samples.   Although the variations of these points from the line are greater than those in Figs. 4 and 5, the agreement is close enough to satisfy the requirements for the clinical determinations of serum proteins.   The time of centrifugation of the samples was not strictly controlled, and no doubt more uniform results would have been obtained if this factor had been kept constant.

<div align="center">SUMMARY</div>

1. Blood serum proteins form biuret color solutions which by analysis on the spectrophotometer give optical densities at 560 mμ that bear a linear relationship with the protein nitrogen deter-

mined by the Kjeldahl procedure. The density values remained practically constant for at least 48 hours. The optical density at 700 mμ of the same solutions decreases with time and the visual tone of the solution changes from purplish red toward the red.

2. Clear biuret color solutions were prepared by introducing 20 per cent copper sulfate solutions into 3 per cent sodium hydroxide solutions of the protein precipitated from the serum by trichloroacetic acid, centrifuging out the excess cupric hydroxide, and filtering the supernatant fluid through Munktell No. 00 paper.

3. Protein nitrogen determinations on these biuret color solutions and on dilutions of the serum in saline or sodium hydroxide show that no protein was removed by the precipitated cupric hydroxide when 20 per cent copper sulfate was used. However, with 2 per cent copper solutions, there is evidence of protein loss.

4. The formation of the biuret color without the previous precipitation of the protein with trichloroacetic acid never gave clear solutions on which good spectrophotometric measurements could be made, and under certain conditions protein was always brought out of solution by the excess copper.

5. Rabbit, dog, and human serum proteins give the same density value at 560 mμ per gm. of protein.

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THE BIURET REACTION IN THE DETERMINATION OF SERUM PROTEINS

II. MEASUREMENTS MADE BY A DUBOSCQ COLORIMETER COMPARED WITH VALUES OBTAINED BY THE KJELDAHL PROCEDURE

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From the results reported in Paper I of this series (1) it is evident that the biuret reaction may be used for the estimation of serum proteins with a high degree of accuracy when the optical density values at wave-length 560 mμ are obtained with a spectrophotometer, and when the color solutions are prepared by the introduction of 20 per cent copper sulfate to protein solutions in which the concentration of alkali is 3 per cent. However, optical instruments which permit the selection of narrow ranges of the spectrum are not available in many laboratories, but in such places the Duboscq type of colorimeter is still widely used for colorimetric methods. The many requests for the application of the information obtained in our work (1) to a very simple procedure that might be used on small samples of sera with the common laboratory equipment led us to determine the reliability of a method under such conditions. The results are such that we believe the method should be called to the attention of many laboratory workers who are anxious to secure reliable protein determinations with the least time and equipment.

The method to be presented is similar to that suggested by Fine (2) in which serum diluted with physiological saline was used as a standard for colorimetric comparison. Fine's statement that the standard solution could be kept for 6 months if preserved with chloroform has been doubted by many workers, because, in their experience, the solutions became turbid in a very short time.

The problem of a suitable standard was solved as far as we were concerned by the use of rabbit sera. Stock solutions prepared from human or dog sera became turbid in some cases after standing for only a week, but in no instance has a dilution of rabbit serum deteriorated so rapidly.   Two dilutions of rabbit sera stored under conditions described below have remained clear and given constant biuret colors for more than a year.   From our previous work we feel that the use of a 20 per cent copper sulfate solution should be much better than the 5 per cent solution as employed by Fine.   The precipitation of the protein by trichloroacetic acid as suggested by Fine is recommended for the production of clear color solutions.   The addition of a dilute solution of copper sulfate to alkaline serum solutions proposed by Kingsley (3) has never given us the clear reproducible solutions that are possible when colors are developed in alkaline solutions of the precipitated protein.   With the Kingsley method we have obtained results comparable to those shown in his paper only under the most rigorous control of the time of reading after production of colors.   In these experiments we never used ether to clear the solutions because none of the sera was jaundiced or lipemic.

Method

Reagents—
0.9 per cent sodium chloride solution.
10 per cent trichloroacetic acid solution.
3 per cent sodium hydroxide solution.
20 per cent copper sulfate ($CuSO_4 \cdot 5H_2O$) solution.

Preparation of Standard—10 cc. of blood serum from a normal rabbit with a total serum protein of about 6 gm. per 100 cc. are diluted to exactly 250 cc. with 0.9 per cent sodium chloride solution.   Total nitrogen concentration of this sample is determined by the micro-Kjeldahl procedure described previously by Robinson, Price, and Cullen (4).   Non-protein nitrogen is determined at the same time on a sample of the serum.   The protein content is obtained by subtracting the non-protein nitrogen from the total nitrogen, and converting the protein nitrogen to protein with the factor 6.25.   The concentration of protein in the diluted serum should be approximately 0.25 per cent.   This stock solution is distributed in 50 cc. containers, preserved with a crystal of thymol, and stored in a refrigerator when not in use.   This diluted rabbit

serum standard usually has a very faint opalescence which interferes in no way with the final color and which remains stable for at least 6 months. Any increase in turbidity or any flocculation has been regarded with suspicion and such standards should be discarded for new ones.

Total Serum Protein—0.2 cc. of serum is measured with a "contain" pipette into a 15 cc. graduated centrifuge tube, the pipette being washed several times with saline solution. If duplicate determinations are desired, one may dilute 0.5 cc. of serum to 10 cc. with saline and then measure two portions of 4 cc. each with a transfer pipette into the centrifuge tubes.

The volume of the diluted serum is brought to about 5 cc. with saline and an equal amount of 10 per cent trichloroacetic acid is is added. The contents are well mixed with a fine glass rod which is rinsed with a little acid when it is removed. Centrifuge the mixture for 5 to 10 minutes, or until the supernatant fluid is clear and can be poured off without disturbing the precipitate. Invert the tube and allow to drain well on a filter paper.

The protein precipitate is mixed with a drop of 3 per cent sodium hydroxide by means of a fine stirring rod until a smooth paste is formed. A few cc. of sodium hydroxide are then added to complete solution of the protein. The mixture must be inspected for small gel-like particles which indicate that the protein is not yet entirely dissolved. After the volume is brought to about 9 cc. with the alkali, 0.25 cc. of 20 per cent copper sulfate is added and the final volume adjusted to 10 cc. with the alkali. Close the tube with a clean rubber stopper, and shake the contents vigorously for a minute in order to develop the biuret color.

After standing for 10 minutes or longer the color solutions are centrifuged at high speed for about $\frac{1}{2}$ hour in order to remove the excess cupric hydroxide. The supernatant liquid is carefully drawn off with a pipette, and compared in a Duboscq colorimeter with a standard color solution. The standard is prepared simultaneously with the unknown by using 5 cc. of standard stock solution in a manner identical with that described for the unknown. If the standard is set at 20 mm. the calculation is

$$\frac{20.0}{\text{Reading of unknown}} \times \frac{\text{gm. protein in standard}}{\text{cc. serum in unknown}} \times 100$$

$$= \text{gm. protein per 100 cc. serum}$$

Serum Albumin—30 parts of 22 per cent sodium sulfate are added to 1 part of serum in order to precipitate the globulin (Howe (5)). The mixtures are filtered or centrifuged at the end of 4 hours in the manner described by us (6). 7 cc. of filtrate are measured into a 15 cc. centrifuge tube and the protein is precipitated with trichloroacetic acid. The procedure is in every detail identical with that described for total serum proteins. However, in order to obtain more equal color comparison, it is preferable

TABLE I

Deviations of Serum Protein Determinations by Biuret Colorimetric Method from Values Calculated from Nitrogen Determinations by Kjeldahl Method

	No. of subjects	No. of determinations	Range of values of protein concentration	Range of deviations between the two methods	Standard deviation* $\sqrt{\dfrac{\Sigma d^2}{N-1}}$
Total serum proteins					
			gm. per 100 cc.	*gm. per 100 cc.*	
Human.................	18	26	3.2–8.4	−0.6 to +0.6	±0.291
Dog....................	10	20	4.7–7.5	−0.3 " +0.5	±0.172
Rabbit.................	8	14	5.3–6.5	−0.2 " +0.4	±0.151
Total.................	36	60			±0.225
Serum albumins					
Human.................	20	25	1.9–5.6	−0.3 to +0.3	±0.171
Dog....................	9	14	2.5–3.7	−0.2 " +0.2	±0.115
Rabbit.................	4	7	3.4–4.9	−0.1 " +0.3	±0.196
Total.................	33	46			±0.156

* *d* is the deviation between the two methods.

to use 4 cc. of the stock rabbit serum solution instead of 5 cc. for the standard color. The calculation of the serum albumin is

$$\frac{20.0}{\text{Reading of unknown}} \times \text{gm. protein in standard} \times \frac{31}{7} \times 100$$

$$= \text{gm. albumin per 100 cc. serum}$$

Results

60 determinations of the total serum protein and forty-six determinations of the serum albumin were made by this method on

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human, dog, and rabbit sera and compared with the protein values calculated from nitrogen determinations by the Kjeldahl method. The human subjects consisted of normal persons and of sick patients. Twenty-two determinations (eleven total serum proteins and eleven serum albumins) of the latter group were on the blood sera of children with the nephrotic syndrome. The dogs and rabbits were apparently normal animals which had not been used for other experimental procedures. The results are summarized in Table I. The greatest deviation between the Kjeldahl and biuret method obtained at any time was 0.6 gm. of protein per 100 cc. of serum. The mean of the deviations for total serum proteins was -0.028 gm. per 100 cc. of serum and for serum albumin was -0.021. These small differences between the two methods were not statistically significant.

SUMMARY

1. A method is described in detail for the determination of serum proteins by the biuret reaction in which the color comparisons are made with the Duboscq colorimeter. The suitability of a dilute rabbit serum saline solution as a standard is discussed.

2. Results by this method show satisfactory agreement with those obtained from Kjeldahl determinations, so that the use of this procedure is warranted for the reliable estimation of serum proteins when the amount of sample, time, and equipment are limited.

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THE KINETICS OF THE ENZYMATIC SYNTHESIS OF GLYCOGEN FROM GLUCOSE-1-PHOSPHATE*

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The reversible enzymatic reaction, glycogen + inorganic phosphate $\rightleftarrows$ glucose-1-phosphate, is of unusual interest, since it is the first known case of an *in vitro* synthesis of a polysaccharide of high molecular weight. In the following pages the kinetics of this reaction are described in some detail; its physiological significance has been discussed in recent articles (1).

When the reaction, catalyzed by the enzyme phosphorylase, proceeds to the left, inorganic phosphate is formed which may be determined by the method of Fiske and Subbarow (2), with the Summerson (3) photoelectric colorimeter. This offers a convenient means of studying the reaction. The amount of polysaccharide formed agrees well with the value calculated from inorganic phosphate liberated (4).[1] Phosphorylase was prepared from mammalian skeletal muscle, heart, brain, and liver. The method of purification of the enzyme was the same as outlined previously (4) but several improvements were introduced which will be described in a later publication.

Substrate Concentration—The curve expressing the relation

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[1] Certain precautions are necessary in the glycogen determination according to Pflueger's method. Glucose-1-phosphate is not destroyed during the protein hydrolysis in strong alkali. When KOH is used, there is some danger that glucose-1-phosphate is included in the glycogen precipitate, because of the low solubility of the potassium salt of this ester in 50 per cent alcohol. This possible source of error is avoided by the use of NaOH instead of KOH and by carrying out a second alcohol precipitation after the first alcohol precipitate of the polysaccharide is dissolved in water.

between initial rate of enzyme activity and glucose-1-phosphate concentration shows a steep rise up to 7 mM and a marked flattening out at concentrations above 12 mM. For example, when the initial glucose-1-phosphate concentration is increased from 14 to 26 mM, the rate of enzyme activity rises only 11.5 per cent. Most experiments reported in this paper were carried out with substrate concentrations above 12 mM and the standard substrate concentration chosen for activity determinations was 16 mM.

The concentration of glucose-1-phosphate at which one-half of the enzyme is saturated with substrate has been calculated by means of Equation 1 (cf. Michaelis and Menten (5)).

$$v = \frac{V_{max.} \times c}{k + c} \tag{1}$$

in which v is the observed velocity of enzyme action and c the glucose-1-phosphate concentration for velocity v. $V_{max.}$ (the velocity for full saturation of the enzyme) is obtained by a graphic method introduced by Lineweaver and Burk (6) in which $1/v$ is plotted against $1/c$ and in which $1/V_{max.}$ is the ordinate intercept; i.e.,

$$\frac{1}{v} = \frac{1}{c} \times \frac{k}{V_{max.}} + \frac{1}{V_{max.}} \tag{2}$$

The value of k determined in this manner was 4.75 for a substrate concentration of 7 mM, 4.8 for 14 mM, and 4.7 for 26 mM; average for $k = 4.75$ mM glucose-1-phosphate. The same method was used for the calculation of the dissociation of the enzyme-adenylic acid complex.

Adenylic Acid—It has been shown that adenylic acid (adenosine-5-phosphate) is necessary for the activity of the phosphorylase in both directions; this coenzyme effect of adenylic acid has been investigated with enzymes prepared from muscle, heart, brain, and liver (7, 4). The difficulty of removing the last traces of adenylic acid from enzyme preparations by dialysis has been emphasized.

In Fig. 1 are reproduced coenzyme-activity curves for a purified and dialyzed enzyme preparation from calf brain. The enzyme was inactive without addition of adenylic acid. The concentration of adenylic acid at which one-half of the enzyme is

combined with adenylic acid (which corresponds to the constant k in Equation 1) may be roughly estimated by inspection of Fig. 1; it is the concentration of adenylic acid which gives one-half of the maximal velocity, the maximal velocity corresponding to full saturation of the enzyme with adenylic acid. When the data of Fig. 1 are analyzed according to Equations 1 and 2, one obtains the following values for k for the first three points of each curve: 5 minutes, 3.2, 3.3, 3.1; 10 minutes, 3.1, 3.2, 3.1; 15 minutes, 2.9, 3.0, 3.1; average 3.1×10^{-5} M adenylic acid (for pH
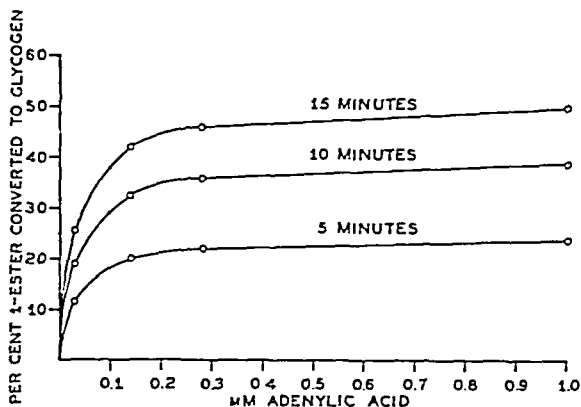
FIG. 1. Effect of adenylic acid on glycogen synthesis in a dialyzed enzyme preparation of calf brain. The reaction mixture contained 15 mM glucose-1-phosphate, 100 mg. per cent of glycogen, 0.05 M veronal buffer of pH 6.9, and varying amounts of adenylic acid. The period of incubation was 5, 10, and 15 minutes, as marked on the curves. The temperature was 25°.

6.9 and 25°). In an experiment with a dialyzed muscle enzyme the average value for k was 2.8×10^{-5} M adenylic acid at pH 6.2 and 3.2×10^{-5} M adenylic acid at pH 7.2.

As pointed out by Warburg and Christian (8), Lipmann (9), and others, Equation 1 (or the equation $k = c\ (V_{max.} - v)/v$ from which Equation 1 is derived) is valid only in cases in which the amount of bound coenzyme is very small in comparison with the amount of free coenzyme. This prerequisite is fulfilled in the case of adenylic acid. A rough estimate which is based on

diffusion measurements[2] indicates that the molecular weight of the enzyme is about 250,000. In a later section it is shown that an activity of 800 to 1000 enzyme units per mg. of protein is obtained in purified enzyme preparations. The enzyme solution used in Fig. 1 contained 24 phosphorylase units per cc., which would correspond to 0.03 mg. of enzyme protein, assuming an activity of 800 units per mg. Hence, it is possible to calculate what percentage of the total adenylic acid is bound when one-half of the enzyme protein is combined with adenylic acid. For a 1:1 ratio one finds

$$\frac{0.6 \times 10^{-7} \text{ M protein} \times 100}{3 \times 10^{-5} \text{ M adenylic acid}} = 0.2\%$$

The adenylic acid-phosphorylase complex ($k = 3 \times 10^{-5}$) is more dissociated than the thiamine pyrophosphate-pyruvic dehydrogenase ($k = 2.7 \times 10^{-6}$) and less dissociated than the diphosphopyridine nucleotide-alcohol dehydrogenase complex ($k = 9 \times 10^{-5}$). The dissociation constant, k, gives an approximate idea of the concentration of adenylic acid which is needed in the intact cell for the activity of the enzyme. Free adenylic acid has not been found in intact muscle, but it seems doubtful whether the methods used were sensitive enough for the detection of adenylic acid in concentrations of the order of 3×10^{-5} M.

Inosinic acid has only a minimal effect on phosphorylase activity. In the following experiment a purified, dialyzed muscle enzyme preparation was used. During 20 minutes of incubation 8 per cent of the added 1-ester was converted to glycogen without addition of nucleotides, 12.2 per cent with 5×10^{-3} M inosinic acid, and 44.7 per cent with 4×10^{-4} M adenylic acid. Similar results were obtained in previous experiments in which the reaction was measured in the other direction (7).

Activating Effect of Glycogen—One of the remarkable properties of·the enzyme is that it is unable to synthesize glycogen unless a small amount of glycogen is added to the reaction mixture (10).[3] It was noted that comparatively crude enzyme preparations of brain, heart, and muscle showed a short lag period (5 to 15 min-

[2] These measurements were carried out by Dr. Gerhard Schmidt.

[3] An analogous observation has recently been made by Hanes (11) for the enzymatic synthesis of starch by plant phosphorylase.

utes) before glycogen synthesis from glucose-1-phosphate set in. Upon purification of these enzymes the lag period became longer and longer until activity in the direction of glycogen synthesis disappeared altogether. Yet, these same enzyme solutions acted without delay in the reverse direction; *i.e.*, they formed glucose-1-phosphate when glycogen and inorganic phosphate were added.[4] Similar experiments with enzyme preparations of liver showed that they had no lag period when crude preparations were used and that they developed a short lag after purifica-
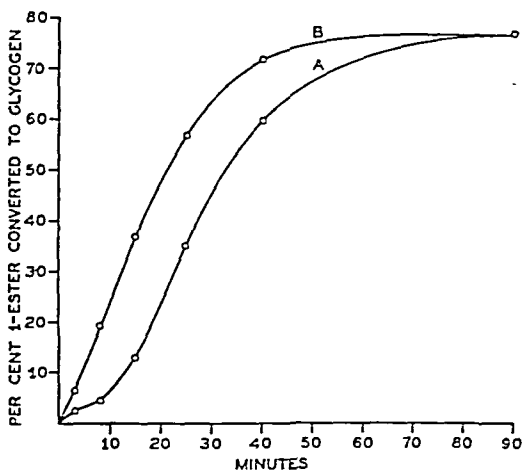
FIG. 2. Effect of glycogen on lag period. A brain enzyme was used. The reaction mixture contained 14 mM glucose-1-phosphate, 1 mM adenylic acid, and 0.05 M glycerophosphate buffer of pH 7. In the experiment shown in Curve A no glycogen was added; in Curve B 1.4 mg. of glycogen per 100 cc. of reaction mixture were added.

tion. A clue to the situation was the observation that enzyme preparations of liver always included some glycogen (owing to the large amount of glycogen which is extracted from this organ by water), while enzyme preparations of other tissues contained

[4] Kiessling (12) observed upon fractionation of the yeast phosphorylase with ammonium sulfate that it lost activity in the direction to the left, while it retained activity in the direction to the right. He drew the erroneous conclusion that two enzymes are involved, one synthesizing glycogen and the other breaking it down, and that he had succeeded in a separation of these two enzymes.

only traces of glycogen. We therefore investigated whether addition of glycogen would abolish the lag period. An experiment of this type with a brain enzyme is shown in Fig. 2. It may be seen that the addition of only 1.4 mg. of glycogen (prepared from rabbit liver by Somogyi's (13) method) per 100 cc. of reaction mixture promptly abolished the lag period. This
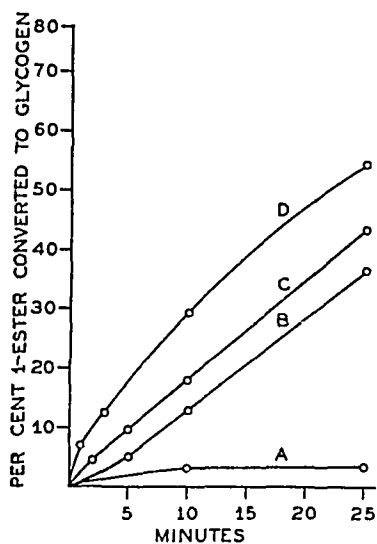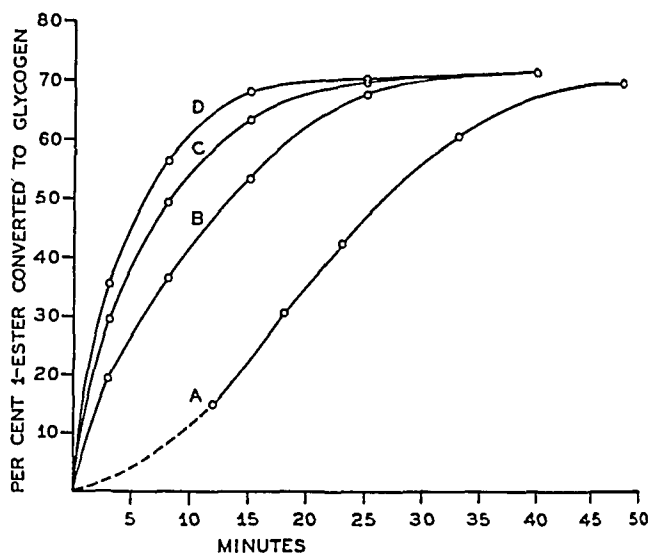
FIG. 3. Activating effect of glycogen. An enzyme preparation of rabbit brain was used. The reaction mixture contained 15 mM glucose-1-phosphate and 1 mM adenylic acid. In the experiment shown in Curve A no glycogen was added; Curve B, 0.6; Curve C, 1.2; and Curve D, 12 mg. of glycogen per 100 cc.

FIG. 4. Effect of increasing amounts of glycogen on activity of brain enzyme. The reaction mixture contained 15 mM glucose-1-phosphate, 1 mM adenylic acid, and 0.05 M glycerophosphate buffer of pH 7.4. In the experiment shown in Curve A no glycogen was added; Curve B, 10; Curve C, 100; and Curve D, 500 mg. of glycogen per 100 cc.

indicates that the S-shaped curve obtained when no glycogen is added is due (a) to traces of glycogen present in the enzyme solution which start enzyme activity at a low rate during the lag period and (b) to the autocatalytic effect of the newly formed glycogen. In Fig. 3 is shown an experiment with an enzyme solution prepared from brain which showed without glycogen addition too low an activity during 1 hour of incubation to reach

the autocatalytic part of the curve. Addition of 0.6 mg. of glycogen per 100 cc. was sufficient to activate the enzyme but not enough to abolish the lag period; addition of 1.2 mg. of glycogen abolished the lag period. Since 12 mg. of glycogen caused a more rapid rate of enzyme action than 1.2 mg., it seemed desirable to test the effect of still larger amounts of glycogen. Fig. 4 shows that the rate of enzyme activity is greater with 100 than with 10 mg. of glycogen per 100 cc. and that a maximal rate is approached when 500 mg. of glycogen per 100 cc. are added. It may also be seen that the position of the equilibrium is not influenced by the glycogen concentration.

Analogous experiments with muscle phosphorylase revealed a quantitative difference as regards the activating effect of small amounts of glycogen. It may be seen in Fig. 5 that when 10 mg. of glycogen per 100 cc. are added (Curve B) there is a marked falling off in rate after a few minutes of activity, so that several hours would be required until equilibrium is reached, and that even 30 mg. of glycogen per 100 cc. (Curve D) are insufficient for a rapid attainment of equilibrium.

The experiment in Fig. 6 was undertaken in order to throw some light on the different behavior of the muscle enzyme when small amounts of glycogen are added. The enzyme was activated by addition of 10 mg. per cent of glycogen and showed a marked falling off in the rate of its action after 20 minutes of incubation, in spite of the fact that at that time the concentration of the newly formed polysaccharide had reached 140 mg. per cent. This shows that the newly formed polysaccharide did not activate a sufficient number of new enzyme molecules to carry the reaction to completion; *i.e.*, it had less "autocatalytic" effect than the polysaccharide formed by brain, heart, and liver enzymes.[5] A further addition of glycogen after 20 minutes of incu-

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[5] It may be recalled in this connection that the latter enzymes synthesize a polysaccharide which like ordinary glycogen gives a brown color with iodine, while muscle phosphorylase (that of rabbit, rat, cat, calf, frog, and carp has been tested) forms a polysaccharide *in vitro* which gives a blue color with iodine, a fact which indicates that it is different from ordinary glycogen (14). This polysaccharide, as well as starch, is converted to glucose-1-phosphate when the reaction is to the right and its addition starts enzyme activity in otherwise inactive enzyme solutions when the reaction is to the left.

bation led to a rapid resumption of enzyme activity so that equilibrium was approached in 10 minutes. This finding indicates that the reaction mixture must have contained enzyme molecules which could not be reached by the newly formed polysaccharide and it implies that each activated enzyme molecule can synthesize only a limited amount of glycogen.[6]
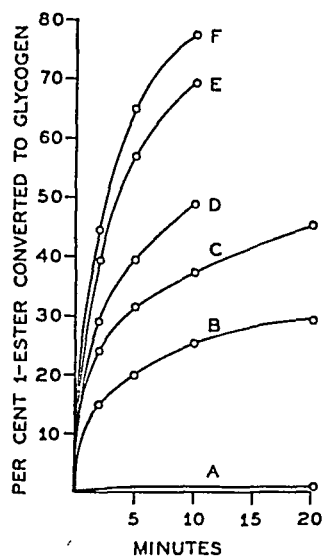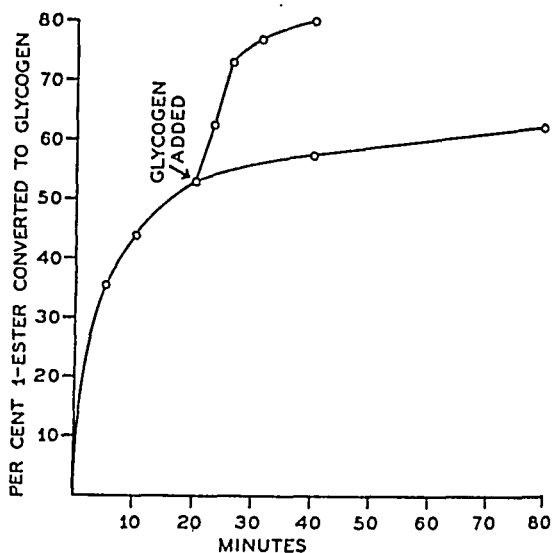
FIG. 5.   FIG. 6.

FIG. 5. Effect of increasing amounts of glycogen on activity of muscle enzyme. The reaction mixture contained 14 mM glucose-1-phosphate, 1 mM adenylic acid, and 0.05 M glycerophosphate buffer of pH 6.3. In the experiment shown in Curve A no glycogen was added; Curve B, 10; Curve C, 20; Curve D, 30; Curve E, 75; and Curve F, 150 mg. of glycogen per 100 cc.

FIG. 6. Effect of a second addition of glycogen on activity of muscle enzyme. The reaction mixture contained 15 mM glucose-1-phosphate, 1 mM adenylic acid, 0.05 M glycerophosphate buffer of pH 6.3 and 10 mg. per cent of glycogen. After 20 minutes of incubation the reaction mixture was divided into two parts to one of which 140 mg. of glycogen per 100 cc. were added, as marked by the arrow.

The following observations with muscle enzymes suggest an explanation for the different behavior of the added and the newly

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[6] This is supported by the observation that when large amounts of substrate (50 mM glucose-1-phosphate) and enough glycogen for full activation of the enzyme are added, the reaction also stops before equilibrium is reached and that it goes to completion when more enzyme is added.

formed polysaccharide; they were made possible by the fact that the two polysaccharides can be distinguished on the basis of their color reaction with iodine. The glycogen which is added does not cause flocculation when mixed with the enzyme solution. The newly formed polysaccharide causes first a turbidity and later flocculation. The centrifuged and washed precipitate appears granular under the microscope and stains deep blue with iodine. Even when washed with 5 per cent trichloroacetic acid, hardly any of the newly formed polysaccharide can be extracted. In order to free the polysaccharide of protein and to make it water-soluble, it is necessary to digest these granules in strong alkali. It is assumed that the newly formed polysaccharide remains bound to the surface of the enzyme, forming the granules just mentioned, and that this limits the amount of polysaccharide which can be synthesized per enzyme molecule and prevents the activation of new enzyme molecules. The polysaccharide formed by brain (or liver and heart) phosphorylase, which does not seem to differ from ordinary glycogen, remains apparently less closely attached to the enzyme protein and therefore has more auto-catalytic activity.

The following represents an analysis of the activating effect of the added glycogen. In Fig. 7, Curve A, is shown the effect of increasing amounts of glycogen on the initial velocity of the reaction. The shape of this curve is similar to that obtained upon addition of increasing amounts of a coenzyme and a straight line is obtained when the same data are plotted according to Equation 2 (Fig. 7, Curve B). The values for the 2 minute incubation period of Fig. 5 were also found to fit a straight line when plotted in this manner. A calculation of the data of these two experiments according to Equation 1 gives reasonably constant values for k; i.e., the values do not deviate from the mean by more than ± 3 per cent (Table I). As has been discussed in the case of adenylic acid, Equation 1 presupposes that only a negligible part of the total amount of coenzyme present in the solution is in the combined form. The concentration of protein in the experiment in Fig. 7 was 15 mg. per 100 cc.,[7] while the con-

[7] Cataphoresis experiments with the Theorell apparatus (15) which will be described in another paper indicate that the enzyme protein has been freed to a considerable extent of other proteins.
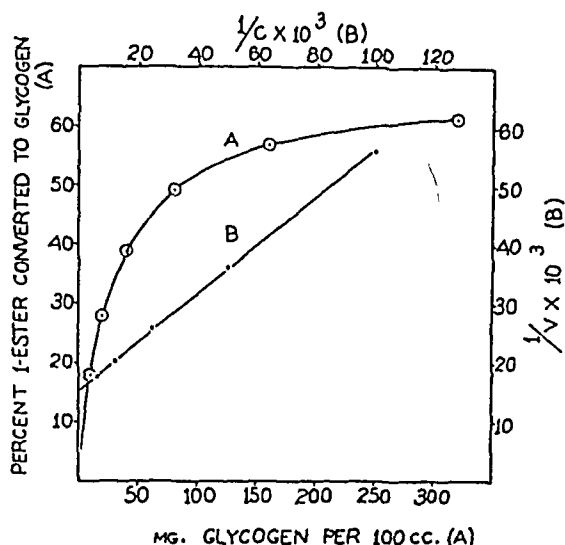
Fig. 7. Coenzyme effect of added glycogen. A muscle enzyme was used. The reaction mixture contained 14 mM glucose-1-phosphate, 1 mM adenylic acid, and 0.05 M glycerophosphate buffer of pH 6.7. The period of incubation was 5 minutes at 25°. In Curve B the data used for the construction of Curve A were plotted as the reciprocals of velocity and of glycogen concentration according to Equation 2.

TABLE I

Calculation of k by Means of Equation 1

c Glycogen added	v 1-Ester converted to glycogen	k_{glycogen}	Remarks
mg. per 100 cc.	*per cent*	*mg. per 100 cc.*	
10	15.2	24.6	Data of Fig. 5, 2
20	24.1	23.6	min. incubation
30	29.2	24.0	period
75	39.3	25.4	
150	44.9	25.7	
	52.6 ($V_{\text{max.}}$)		
Average............		24.7 ± 0.8	
10	17.9	27.2	Data of Fig. 7
20	27.8	27.9	
40	38.9	28.5	
80	49.3	28.1	
160	56.9	27.3	
320	61.4	27.1	
	66.6 ($V_{\text{max.}}$)		
Average............		27.6 ± 0.5	

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centration of glycogen corresponding to k (*i.e.* for half saturation of the enzyme) was 27.6 mg. per 100 cc. These figures indicate that a large percentage of the glycogen (assuming for it a molecular weight $>250,000$) would be bound if 1 enzyme molecule were to combine with 1 glycogen molecule, in which case Equation 1 could not apply. The fact that the enzyme-glycogen relationship conforms to Equation 1 argues in favor of the assumption that glycogen combines as a multiple unit.

When one attempts to form a concept of the mechanism of the enzymatic synthesis of glycogen (and of starch), one is faced by the following problem. It has been shown that glycogen is made up of a large number of units, each unit consisting of 12 (or 18) glucose molecules which form a maltosidic chain. The formation of one of these units on the surface of 1 enzyme molecule is conceivable, but it is difficult to see how 1 enzyme molecule can put together a hundred or more of these units to form a polysaccharide of high molecular weight. The following working hypothesis is presented. It is assumed that many enzyme-adenylic acid molecules (which are oriented by an activating glycogen molecule and so form the catalytically active complex) are concerned in the linking together of units, the resulting structure being a glycogen or starch granule.

Effect of Glutathione—Gill and Lehmann (16) observed in experiments with aged muscle extracts that reduced glutathione as well as other reducing agents increased the formation of hexose-6-phosphate. The formation of this ester involves three enzymatic reactions; namely, glycogen $+$ inorganic phosphate $\rightleftharpoons$ glucose-1-phosphate $\rightarrow$ glucose-6-phosphate $\leftrightarrows$ fructose-6-phosphate. It was later reported by Lehmann (17) that glutathione had no effect on the first step, while it markedly accelerated the second step. This accelerating effect on phosphoglucomutase, the enzyme which catalyzes the second step, has been confirmed in this laboratory (unpublished experiments). A purified enzyme was used which was free of phosphorylase and of the enzyme which catalyzes the third step.

A possible effect of glutathione on phosphorylase activity was reinvestigated with purified enzyme solutions prepared from muscle and brain tissue. The experiment in Fig. 8 shows that glutathione accelerates the enzymatic synthesis of glycogen and

that this effect is stronger at pH 7.4 than at pH 6.2.   In comparing different glutathione concentrations it was found that 0.02 was as effective as 0.04 M, while 0.01 M was slightly less active. The position of the equilibrium of the reaction was not influenced by glutathione.

In these experiments glutathione was added to the enzyme simultaneously with the substrate.   When the enzyme solution was allowed to stand for some time in the presence of glutathione,
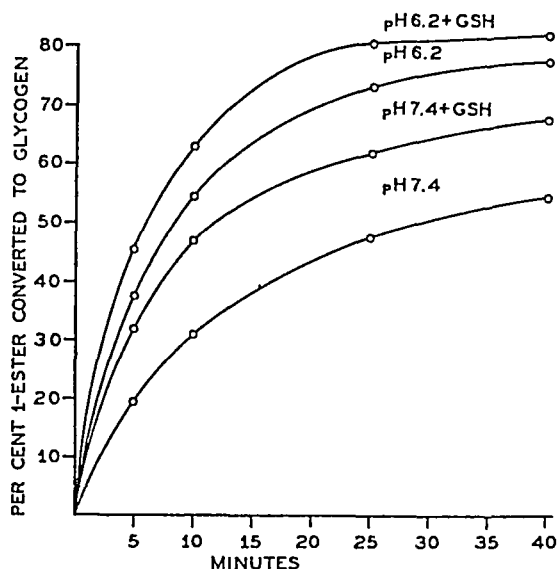
FIG. 8. Effect of glutathione on muscle enzyme at two different hydrogen ion concentrations.   The reaction mixture contained 16 mM glucose-1-phosphate, 1 mM adenylic acid, 200 mg. per cent of glycogen, and 0.1 M glycerophosphate buffer.   Glutathione (0.02 M), adjusted to the same pH as the buffer, was added simultaneously with the substrate to one part of the reaction mixture.

the activity was greater than when glutathione was added with the substrate.   A time factor is therefore involved in the reaction between glutathione and the enzyme.   Treatment of the enzyme with H_2S increases its activity but denaturation of the protein occurs after some time.

Cu^{++} in a concentration of 5×10^{-4} M inhibits the enzyme completely and this inhibition is prevented by addition of 0.04 M glutathione.   It is generally assumed that enzymes which are

inhibited by small concentrations of Cu^{++} and activated by —SH compounds and other reducing agents possess a sulfhydryl group (or groups) which is important for their activity. Further investigation seems necessary before this can be accepted as true in the case of the phosphorylase.

There is still another phenomenon which deserves mention, especially in conjunction with the findings reported in the preceding section. The presence of glutathione prevents or greatly delays the flocculation which occurs during enzyme activity. This effect is more marked at slightly alkaline or neutral than at acid reaction and appears to be parallel to the accelerating effect of glutathione on the reaction.

Equilibrium—The enzyme preparations of muscle, liver, and brain used in these experiments were sufficiently purified to free them of phosphoglucomutase, the enzyme which converts glucose-1- to glucose-6-phosphate (18). In the case of liver enzyme preparations it was also necessary to make sure that no phosphatase was present. Both these enzymes, by removing glucose-1-phosphate, prevent the attainment of a true equilibrium of the reaction, glycogen + inorganic phosphate ⇌ glucose-1-phosphate. As pointed out before, an effect of glycogen (in concentrations of 10 to 500 mg. per cent) on the ratio of inorganic phosphate to glucose-1-phosphate at equilibrium could not be demonstrated.[8]

It is shown in this section that the position of the equilibrium is influenced by the pH of the reaction mixture. 0.05 M glycerophosphate or veronal-acetate buffers were used in order to prevent the shift in pH resulting from the conversion of glucose-1-phosphate (pK'_2 6.13) to orthophosphate (pK'_2 6.83) or *vice versa*. In many cases the pH of the reaction mixture was measured by means of a glass electrode at the start and at the end of the ex-

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[8] This may be interpreted as follows: n moles of glucose-1-phosphate → n moles of inorganic phosphate + 1 mole of glycogen.

$$\frac{[\text{Glucose-1-phosphate}]^n}{[\text{Glycogen}] \times [\text{inorganic phosphate}]^n} = k \text{ (true equilibrium constant)}$$

$$\text{Ratio } \frac{\text{glucose-1-phosphate}}{\text{inorganic phosphate}} = \sqrt[n]{k} \times \sqrt[n]{\text{glycogen}} = k' \times \sqrt[n]{\text{glycogen}}$$

For a molecular weight of glycogen of 180,000, $n = 1000$. Changing the concentration of glycogen 100-fold would affect the value of the ratio less than 1 per cent.

periments and it was shown that the buffering capacity of the solutions was sufficient to prevent a shift in pH.

In order to be sure that a state of equilibrium had been reached, aliquots of the reaction mixture were analyzed 45 and 60 minutes after the start of the experiments and only those cases were included in Fig. 9 in which there occurred no detectable change in the concentration of inorganic phosphate and glucose-1-phosphate during the last 15 minutes of incubation. Just before the last sample was removed for analysis, the pH of the reaction mixture was measured.
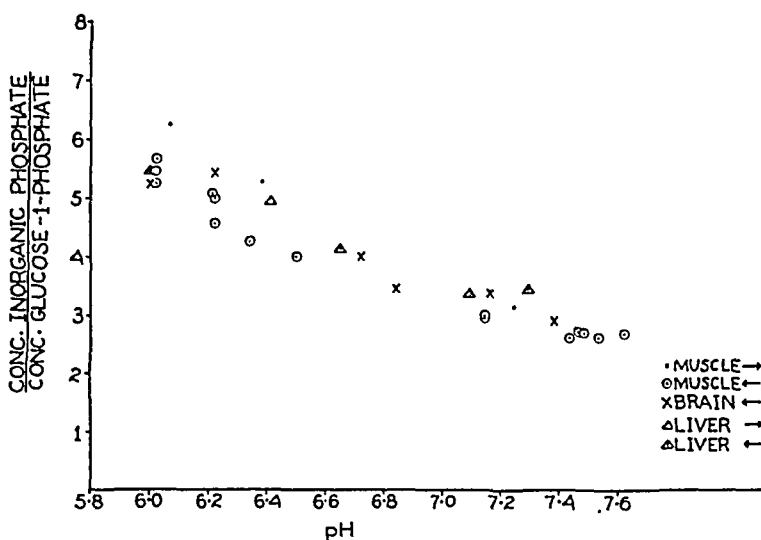
Fig. 9. Effect of pH on the position of the equilibrium of the reaction, glycogen + inorganic phosphate ⇄ glucose-1-phosphate. The arrows on the chart show the direction from which equilibrium was reached.

The data in Fig. 9 show the effect of pH on the position of the equilibrium; they were obtained with enzyme preparations of different organs and with the reaction starting from either side. At pH 6.0 the average ratio of concentrations of inorganic phosphate to glucose-1-phosphate was 5.7, while at pH 7.55 this ratio was 2.9. Intermediate values for this ratio were obtained between pH 6 and 7.5. In a number of experiments small amounts of acid or alkali were added to the reaction mixture after equilibrium had been established at a given pH. The resulting changes in the position of the equilibrium were those to be predicted from the data in Fig. 9; one representative experiment is shown in Table II.

TABLE II

Effect of Addition of Alkali and of Acid on Equilibrium

Time	pH	1-Ester converted to inorganic P	$\dfrac{\text{Inorganic P}}{\text{1-Ester P}}$	Remarks
min.		*per cent*		
5		57.5		
15		76.1		
45		83.8		
71	6.21	83.8	5.17	
76				NaOH added
86		75.1		
106	7.62	73.5	2.77	
115				HCl added
145	6.38	82.6	4.75	

TABLE III

Effect of pH on Value of C in Equation 3

pH	C
6.0	85
6.25	83
6.5	81
6.75	79
7.0	77
7.3	75
7.6	73

TABLE IV

Calculation of Velocity Constant, K, According to Equation 3

Brain enzyme (pH 6.7)			Liver enzyme (pH 7.1)			Muscle enzyme (pH 6.5)		
t	*x*	$K \times 10^3$	*t*	*x*	$K \times 10^3$	*t*	*x*	$K \times 10^3$
min.			*min.*			*min.*		
5	12.4	14.6	5	11.3	14.0	2	9.6	27.5
10	22.4	14.3	10	21.1	14.1	5	21.9	27.4
15	31.5	14.5	21	37.8	14.2	10	37.7	27.2
25	45.3	14.5	30	48.2	14.0	15	49.6	27.3
50	65.1	14.6	42	56.8	14.2	20	58.1	27.4
70	75.1		100	74.9		26	65.2	27.3
∞	80.0		∞	76.0		45	77.9	
						∞	81.0	

An effect of temperature on the position of the equilibrium could not be detected. After equilibrium had been reached at

27° and pH 6, the reaction mixture was cooled down to 14° and this caused no demonstrable shift in the equilibrium.

Unimolecular Reaction Rate—When enough glycogen and preferably also glutathione are added to the enzyme, the reaction, glycogen + inorganic phosphate $\rightleftarrows$ glucose-1-phosphate, is first order in either direction. With muscle enzymes the glycogen concentration has to be at least 500 mg. per cent, while with brain and liver enzymes 200 mg. per cent are sufficient.

The reaction to the left is expressed by the equation

$$K = \frac{1}{t} \log \frac{C}{C - x} \tag{3}$$

where C is the amount of inorganic P present when the reaction reaches equilibrium and x the amount of inorganic P present at time t, both expressed in per cent of the glucose-1-phosphate P present at time t_0. C varies with the pH of the reaction mixture in the manner shown in Table III. In Table IV is given one example each for a brain, liver, and muscle enzyme and it may be seen that the values for the velocity constant, K, show good agreement in each case when calculated according to Equation 3.

Activity Determination—The standard conditions adopted for activity determinations are as follows: neutralized glutathione (0.02 M) is added to the enzyme 30 minutes before activity is measured. The reaction mixture contains glucose-1-phosphate 0.016 M (0.5 mg. of P per cc. of reaction mixture), adenylic acid 0.001 M, glycogen 0.5 per cent, glutathione 0.02 M, glycerophosphate buffer 0.05 M, pH 6.65 (so that $C = 80$; *cf.* Table III);[9] the temperature is kept at 25°.[10] After 5 and 10 minutes of incubation the inorganic P is determined in an aliquot of the reaction mixture. Another aliquot is hydrolyzed for 5 minutes in N sulfuric acid at 100°; the inorganic P found after hydrolysis corresponds to the amount of glucose-1-phosphate which was added. The inorganic P formed is expressed in per cent of the

[9] If another pH is chosen, the appropriate value for C shown in Table III is substituted. The reaction velocity varies with the pH as follows: If the rate at pH 6.5 is designated as 100, it is 83 at pH 6, 98 at pH 6.25, 92 at pH 6.75, 75 at pH 7, and 60 at pH 7.3.

[10] The enzyme shows a normal temperature coefficient between 10–25°. Between 25–40° the temperature coefficient is low owing to partial inactivation of the enzyme during activity.

glucose-1-phosphate P which was added. Small deviations (of the order of ± 2 mM) from the standard glucose-1-phosphate concentration may be corrected for with sufficient accuracy as follows:

$$\% \text{ inorganic P formed} \times \frac{\text{1-ester concentration found}}{\text{standard 1-ester concentration}}$$

This correction is not accurate enough for larger deviations. If the reaction approaches equilibrium during the 10 minute incubation period, the activity determination has to be repeated with a more dilute enzyme solution. The activity is expressed in enzyme units which are calculated by means of Equation 3 so that $K \times 10^3 =$ enzyme units per cc. of reaction mixture. Example: it is found in an activity determination under standard conditions that 22.5 per cent of the added 1-ester is converted to inorganic phosphate in 5 minutes and 40 per cent in 10 minutes; therefore $1/5 \log 80/80 - 22.5 = 0.0287$ and $1/10 \log 80/80 - 40 = 0.0301$. For convenience these values are multiplied by 1000; $i.e.$, 28.7 and 30.1 enzyme units per cc. of reaction mixture. Enzyme units can be calculated in this way or they can be read off directly from a graph in which per cent of inorganic P formed has been plotted against enzyme units according to Equation 3, with a value of 80 for C. Since Equation 3 gives a theoretical curve for a reversible reaction of the first order, it is obvious that the accuracy of the activity determination will be decreased if the reaction is not unimolecular.

An activity of 800 to 1000 units per mg. of protein has been found in purified enzyme preparations of muscle. In such enzyme solutions the initial rate of activity corresponds to the formation of 3.5 mg. of glycogen per mg. of protein per minute.

There exists another relationship between activity and enzyme concentration which can be used to advantage in certain types of experiments. It has been shown previously (19) that when the enzyme is breaking down glycogen the time to phosphorylate a given amount of glycogen is inversely proportional to the enzyme concentration. The same result has been obtained when the enzyme is synthesizing glycogen from glucose-1-phosphate, as shown in Table V. It may be seen that enzyme concentration 1:2 required twice as long and enzyme concentration 1:4 four times as long to synthesize a given amount of glycogen as enzyme concentration 1. This relationship holds whether the reaction

rate is unimolecular (as for the brain enzyme) or whether the rate falls off more rapidly than corresponds to a unimolecular reaction (as for the muscle enzyme).

TABLE V

Effect of Enzyme Dilution on Rate of Glycogen Synthesis

Values are given in per cent of added 1-ester converted to glycogen.

Time	Muscle enzyme			Brain enzyme		
	Relative enzyme concentrations					
	1	1:2	1:4	1	1:2	1:4
min.						
5	46.6			33.9		
10	63.2	45.5		53.3	33.6	
20		63.0	46.2		53.0	33.6
40			62.1			54.1
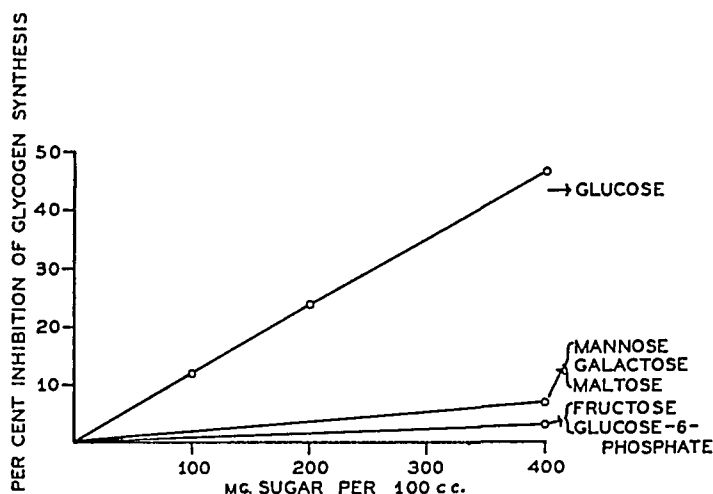
FIG. 10. Inhibitory effect of glucose and other sugars on glycogen synthesis from glucose-1-phosphate. A cat liver enzyme was used. The reaction mixture contained 14 mM glucose-1-phosphate, 1 mM adenylic acid, and varying amounts of sugar. The period of incubation was 10 minutes at 25°.

Effect of Glucose and Other Sugars—It has been shown by Gill and Lehmann (16) that the activity of phosphorylase is inhibited by addition of glucose. A variety of other sugars which have been tested either had no effect or a much weaker inhibitory ac-

tion than glucose.   In all these cases the reaction was studied in the direction to the right (19).

From what is known about reversible enzymatic processes, it was to be expected that glucose would also inhibit the above reaction in the direction to the left.   That this is actually the case is shown in Fig. 10.   It may be seen that the inhibitory effect is proportional to the glucose concentration, being twice as strong with 0.2 as with 0.1 per cent glucose.   In this experiment the

TABLE VI

Competition between Glucose and Glucose-1-Phosphate

Experiment No.	Initial concentrations			Time	1-Ester converted to glycogen	Inhibition of enzyme activity
	Adenylic acid	1-Ester	Glucose			
	mM	mM	mM	min.	per cent	per cent
1	1	19		5	38.4	
	1	19		10	51.4	
	1	19	17	5	26.3	31
	1	19	17	10	36.2	30
	1	37		5	23.4	
	1	37		10	32.7	
	1	37	17	5	19.5	17
	1	37	17	10	26.8	18
2	1	14		5	47.4	
	1	14		10	62.5	
	1	14	17	5	26.9	43
	1	14	17	10	38.8	38
	10	14		5	52.5	
	10	14		10	66.0	
	10	14	17	5	30.5	42
	10	14	17	10	42.7	36

initial 1-ester concentration was kept constant, while the glucose concentration was varied.   An experiment with a constant glucose concentration and different 1-ester concentrations is shown in Table VI.   When glucose and glucose-1-phosphate were present in about the same molar concentrations, the inhibition of enzyme activity amounted to 31 per cent for the 5 minute period, while with the glucose-1-phosphate concentration twice that of glucose the inhibition was only 17 per cent.

These results are best explained by assuming that glucose com-

petes with glucose-1-phosphate (or in the reverse reaction, with inorganic phosphate) for the same active center of the enzyme; *i.e.*, glucose can be displaced by increasing the glucose-1-phosphate concentration and *vice versa*. The relative affinities may be estimated as follows (Table VI): When α,β-glucose (17 mM) was present in. about the same concentration as glucose-1-phosphate (19 mM), 10 minutes were required for the conversion of 36 per cent of the added 1-ester to glycogen, while without glucose it took only 5 minutes to reach the same percentage of conversion. As shown in a previous section this means that only one-half as much enzyme was active in the former as in the latter case; hence α,β-glucose and glucose-1-phosphate have about equal affinities for the enzyme. On this basis one would expect that with the glucose concentration one-half of that of glucose-1-phosphate, two-thirds of the enzyme would remain active; *i.e.*, that it would take 7.5 minutes to reach the same percentage of conversion as is reached by the enzyme without addition of glucose in 5 minutes. This was found to be the case in the experiment in Table VI, when graphic interpolation was used. As a control procedure another factor was varied; namely, the adenylic acid concentration (Experiment 2, Table VI). It was increased from 0.001 to 0.01 M but the glucose inhibition was the same at the low as at the high adenylic acid concentration.

Glucose does not influence the position of the equilibrium of the reaction; it merely prolongs the time required to reach equilibrium. This was to be expected if the action of glucose consisted merely in blocking part of the enzyme molecules, so that they could react neither with glucose-1-phosphate nor with inorganic phosphate.

The experiments with other sugars show (see Fig. 10) that fructose, mannose, galactose, glucose-6-phosphate, and maltose have hardly any inhibitory effect in concentrations in which glucose is strongly inhibitory. This indicates that when the configuration of carbon atoms 2 (fructose and mannose), 4 (galactose), and 6 (glucose-6-phosphate) is different from that of glucose, the affinity for the enzyme is lost.

Glucose-1-phosphate has been shown (20) to have α configuration and it was therefore of interest to compare the inhibitory action of freshly dissolved α- and β-glucose. Owing to the pres-

ence of accelerating ions, mutarotation is very rapid in the reaction mixture, being nearly completed in 10 minutes, but it can nevertheless be shown that during the first 5 minutes of enzyme activity α-glucose is twice as inhibitory as β-glucose. This suggests that β-glucose has very little, if any, inhibitory action on the enzyme.

Phlorhizin—Another agent which inhibits the enzyme activity in both directions is phlorhizin and its aglycone phloretin. This

TABLE VII

Phlorhizin Inhibition

Experiment No.	Initial concentrations			Time	1-Ester converted to glycogen	Inhibition
	Adenylic acid	1-Ester	Phlorhizin			
	$m M$	$m M$	$m M$	min.	per cent	per cent
1	1	19		5	31.6	
	1	19		10	42.2	
	1	19	2.6	5	18.7	41
	1	19	2.6	10	26.2	38
	1	37		5	20.0	
	1	37		10	27.1	
	1	37	2.6	5	12.1	40
	1	37	2.6	10	17.7	35
2	0.25	14		5	39.3	
	0.25	14		10	50.7	
	0.25	14	2.6	5	18.3	53
	0.25	14	2.6	10	27.6	46
	2.5	14		5	42.3	
	2.5	14		10	55.1	
	2.5	14	2.6	5	25.4	40
	2.5	14	2.6	10	37.0	33

inhibition is different from that produced by glucose; *i.e.*, it is not due to a competition with glucose-1-phosphate for the enzyme. In Table VII it may be seen that the phlorhizin inhibition remains the same whether the initial glucose-1-phosphate concentration is 19 or 37 mM, while in the case of glucose the inhibition is less at the high than at the low glucose-1-phosphate concentration (see Table VI). On the other hand, when the adenylic acid concentration is increased (which has no effect on the glucose inhibition), the inhibitory action of phlorhizin is smaller at the high

than at the low adenylic acid concentration, as shown in Experiment 2, Table VII. This suggests that phlorhizin inhibits the activity of the phosphorylase by blocking that part of the enzyme which associates with adenylic acid in the formation of the enzyme-coenzyme complex.

SUMMARY

1. The activity of the enzyme phosphorylase which catalyzes the reversible reaction, glycogen + inorganic phosphate $\rightleftarrows$ glucose-1-phosphate, has been studied, with highly purified muscle as well as purified brain, heart, and liver enzymes. Adenylic acid and glycogen are essential components of the system not only for the reaction to the right, but also to the left. Inosinic acid cannot replace adenylic acid as coenzyme.

2. The dissociation of the enzyme-substrate and the enzyme-coenzyme complex is expressed by the equation of Michaelis and Menten. The concentrations at which the enzyme acts at one-half its maximal velocity, corresponding to the constant k in this equation, are 4.8×10^{-3} M glucose-1-phosphate and 3×10^{-5} M adenylic acid (for pH 6.9 and 25°).

3. Crude enzyme preparations of brain, heart, and muscle show a lag period before activity to the left sets in. Upon purification the activity to the left disappears completely, while full activity is retained to the right. This is not the result of a separation into two enzymes, one synthesizing glycogen and the other breaking down glycogen, as has been assumed by Kiessling. The activity to the left is completely restored when glycogen is added. The S-shaped curve obtained with crude enzyme solutions is due to the presence of traces of glycogen which start activity at a slow rate during the lag period and to the autocatalytic effect of the newly formed polysaccharide.

4. Addition of 1 to 5 mg. of glycogen per 100 cc. abolishes the lag period, but does not maintain enzyme activity in the case of muscle enzymes. This is due to the fact that the polysaccharide formed by muscle enzymes, which gives a blue color with iodine, has less autocatalytic effect than the polysaccharide formed by brain, heart, and liver enzymes, which gives a brown color with iodine. The muscle polysaccharide remains combined with the enzyme and precipitates in the form of microscopic granules which are water-insoluble and stain deep blue with iodine. The

amount of polysaccharide which can be synthesized per enzyme molecule and the activation of new enzyme molecules are thus limited.

5. The rate of enzyme activity (to the left) rises with the addition of increasing amounts of glycogen and approaches a maximum at a glycogen concentration of 500 mg. per cent in the case of muscle and of 200 mg. per cent in the case of the other enzymes. An analysis of the activating effect of the added glycogen suggests that one is dealing with a combination of many enzyme molecules with an activating glycogen molecule. The hypothesis is presented that this macromolecule is the catalyst on whose surface the polysaccharide is formed.

6. Reducing agents (0.02 M glutathione, H_2S) increase the activity of purified phosphorylase. In order to obtain the full effect it is necessary to allow the reducing agent to act on the enzyme before the substrate (glucose-1-phosphate) is added. The inhibitory effect of low concentrations of Cu^{++} ions is counteracted by these reducing agents. Glutathione prevents or greatly delays the flocculation which occurs in enzyme solutions of muscle during activity.

7. The position of the equilibrium of the reaction is influenced by the hydrogen ion concentration. The ratio of concentrations of inorganic phosphate to glucose-1-phosphate at equilibrium (which may be reached from either side) is 5.7 at pH 6 and 2.7 at pH 7.6 with intermediate values at intermediate hydrogen ion concentrations. The position of the equilibrium can be shifted back and forth by the addition of acid or alkali. Temperature (between 15–30°), glycogen concentration, reducing agents, and inhibitory substances (glucose, phlorhizin) have no effect on the position of the equilibrium. The pH optimum for the reaction to the left is between 6.3 and 6.5.

8. Under optimal conditions (high initial glycogen concentration, presence of reducing agents) the reaction rate is unimolecular. This permits the enzyme concentration to be expressed in units which are based on a determination of the velocity constant under standard conditions. Over a considerable range of dilution the time required for the synthesis of a given amount of glycogen is inversely proportional to the enzyme concentration. This relationship holds whether or not the reaction rate is unimolecular and may be used as an approximate measure of enzyme concentration.

9. Glucose inhibits phosphorylase activity in either direction. By varying the relative concentrations of glucose and glucose-1-phosphate it is shown that this inhibition is competitive. Although mutarotation is rapid, it can be shown that α-glucose is much more inhibitory than β-glucose; this is of interest in view of the α configuration of glucose-1-phosphate. Fructose, mannose, galactose, glucose-6-phosphate, and maltose have hardly any inhibitory effect in concentrations in which glucose is strongly inhibitory. Phlorhizin and phloretin inhibit the activity of the enzyme in either direction. The phlorhizin inhibition is counteracted by an increase in the adenylic acid concentration, but not by an increase in the glucose-1-phosphate concentration.

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STUDIES ON KETOSIS

XVIII. AN EXPERIMENTAL STUDY OF THE VAN SLYKE PROCEDURE FOR THE DETERMINATION OF β-HYDROXYBUTYRIC ACID*

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The classical procedure of Van Slyke (1) for the determination of β-hydroxybutyric acid and the other acetone bodies has been almost universally employed for the estimation of the ketone bodies in urine and blood. However, earlier investigations from this laboratory (2) have indicated that the factor for calculation of β-hydroxybutyrate from the weight of the mercury precipitate as given by Van Slyke is too low. In the present study we have carried out a series of experiments designed to establish such values with highly purified preparations of β-hydroxybutyric acid.

The sample of l-β-hydroxybutyrate was prepared according to the procedure of Blunden (3). The calcium-zinc double salt of dl-β-hydroxybutyric acid was made from ethyl β-hydroxybutyrate[1] for purposes of purification. Both of these salts were recrystallized several times from water to obtain maximum purity. These preparations had the following constants.

	Found	Theory
Ca-Zn l-β-hydroxybutyrate		
$[\alpha]_D^{18}$ ($l = 2$, $\alpha = -1.57°$, $c = 4.82$)	$-16.28°$	$-16.26°$*
Ash (CaO + ZnO) (average of 4 tests)	25.57	25.55
Ca-Zn dl-β-hydroxybutyrate		
$[\alpha]_D^{18}$ ($l = 2$, $\alpha = 0.00°$, $c = 5.64$)	$0.00°$	$0.00°$
Ash (CaO + ZnO) (average of 4 tests)	26.65	26.55

* See reference (4).

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[1] Prepared from ethyl acetoacetate by high pressure hydrogenation by Dr. R. Dressler.

TABLE I

Factors for Calculation of β-Hydroxybutyrate from Mercury Precipitates As Determined with Pure Ca-Zn Salts of l- and dl-β-Hydroxybutyrate and Ethyl dl-β-Hydroxybutyrate

Quantity in each test, mg.		Weights of mercury ppts., mg.		Per cent recovery on factor 8.45	Per cent oxidized to acetone	Factor showing mg. ppt. for 1 mg. hydroxybutyrate
As salt	As β-hy-droxy-buty-rate	Individual samples	Aver-age			
Ca-Zn l-β-hydroxybutyrate						
16.0	12.9	122.1, 122.4, 123.4, 121.6, 121.7, 121.9, 122.8, 122.4, 121.9, 121.3	122.0	112.1	84.0	9.47
20.0	16.1	152.6, 153.2, 153.9, 153.5, 152.1, 153.2, 154.0, 154.8, 154.5, 151.9	153.4	112.8	84.7	9.53
20.1	16.1	153.9, 153.6, 154.2, 154.8, 153.6, 152.7, 153.4, 154.1, 153.9, 152.0	153.6	112.7	84.6	9.53
Average..........................				112.5	84.5	9.51
Ca-Zn dl-β-hydroxybutyrate						
20.0	16.1	150.3,* 147.7,* 158.0, 157.0, 156.8, 159.4, 154.9, 155.4, 159.7, 154.4	157.0	115.3	86.5	9.75
20.0	16.1	156.2, 154.2, 152.0, 154.5, 152.1, 156.0, 152.3, 154.7, 155.8, 157.5	154.5	113.6	85.2	9.60
19.1	15.4	149.0, 150.4, 149.8, 146.8, 147.5, 151.6, 148.9, 150.5, 147.4, 150.5	149.2	114.7	86.0	9.69
Average..........................				114.5	85.9	9.68
Ethyl dl-β-hydroxybutyrate						
24.5	19.3	195.1, 191.3, 195.5, 193.5, 186.3,* 191.2, 190.0, 196.3, 193.9	193.8 / 185.9†	114.0	85.4	9.62

* Not considered in the average.

† Determined as total acetone; correction made for a small amount of acetoacetic acid.

As an additional confirmation of our results, determinations were also made on a sample of ethyl dl-β-hydroxybutyrate which contained traces of ethyl acetoacetate.[2] The results obtained in these determinations are presented in Table I.

These results were further confirmed by some of us (5) with estimations carried out on the silver salt of l-β-hydroxybutyric acid isolated from the urine of rats fed sodium deutero caproate.

The value for the gm. of mercury precipitate equivalent to 1 gm. of l-β-hydroxybutyrate which we have found, i.e. 9.51, is considerably higher than the factor, 8.45, reported by Van Slyke (1). It seems probable that the higher results are partly to be ascribed to the greater purity of our product, as determined by specific rotation. Investigations in this laboratory indicate that the Ca-Zn salt of crotonic acid may contaminate these preparations to varying degrees if conditions are not properly controlled. Small quantities of such contaminating substances influence the ash analysis only slightly, while the specific rotation and reaction with Denigès'[1] reagent are lowered according to the degree of the impurity. This is a better explanation for lowered values for specific rotation of the Ca-Zn salt in the presence of practically theoretical values for ash analysis than the partial racemization. Another possible source of error has been eliminated by the use of a reflux system incorporating all-glass joints. Since the completion of these results, similar values have been obtained on a Ca-Zn salt by Professor D. R. Drury and Dr. A. N. Wick.[3] Jowett and Quastel (6) have also reported a value of 9.85 for the conversion factor of β-hydroxybutyrate (presumably the dl salt).

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[2] Prepared by Mr. Paul Fischer of the Department of Chemical Engineering.

[3] Personal communication.

PLANT PROTEASES

I. ACTIVATION-INHIBITION REACTIONS

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The recent work of Balls and Lineweaver (1) with crystalline papain supports the view that one or more sulfhydryl groups are essential to the activity of this enzyme. These investigators have adopted the term papainases to characterize the various plant proteolytic enzymes which resemble papain in their reversible activation and inhibition reactions.

It is commonly stated that the properties of bromelin of pineapple are the same as those of papain, although no thorough study of the former enzyme has been made. In the present paper, the nature of the active groups of bromelin is investigated with the aid of reagents that have come into recent use for this purpose. In addition, the activation-inhibition reactions of two other proteases are described. One of these enzymes is from the horsenettle, *Solanum elaeagnifolium*, whose milk-clotting action was first studied by Bodansky (2). The name *solanain* is suggested for this enzyme.[1] The other protease, hitherto unreported, is from the latex of the milkweed, *Asclepias mexicana*. We have recently reported on the properties of the protease from a different milkweed, *Asclepias speciosa* (3), and in this paper these enzymes are distinguished by the names *asclepain m* and *asclepain s*.

Certain experiments with papain and asclepain s, designed to test the sulfhydryl theory, are also described. It will be shown that bromelin, asclepain *m*, and asclepain *s* are papainases, but that solanain gives none of the reactions suggestive of the presence of an active sulfhydryl, and accordingly, the last enzyme is not a papainase.

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[1] It is suggested by Dr. H. Lineweaver and by us that the ending "ain" be used to form the generic names of new proteases from plant sources.

Isolation of Proteases

Bromelin—To obtain this enzyme, 3 liters of juice from fresh pineapple fruit[2] were filtered with the aid of super-cel, and adjusted to pH 6 with ammonia.   Then solid $(NH_4)_2SO_4$ was added to the point of saturation.   The resulting precipitate of crude enzyme was centrifuged down, collected on a Buchner filter, and washed with 0.6 saturated $(NH_4)_2SO_4$.   The precipitate was then redissolved in a liter of 0.02 M NaCN (pH 6), and the solution again made 0.6 saturated with $(NH_4)_2SO_4$.   The precipitate, collected as before, was drained as dry as possible, and redissolved in 600 ml. of 0.02 M NaCN.   Then the enzyme was precipitated by the addition of 3 volumes of acetone.   It was centrifuged down, collected on a Buchner filter, washed with acetone followed by ether, and finally dried in a vacuum desiccator.   The almost colorless, dry product that resulted weighed 5 gm.

Asclepain m—10 ml. of latex from *Asclepias mexicana*[3] were extracted with 10 ml. of 0.05 M NaCN (pH 7), and the filtrate saturated with solid $(NH_4)_2SO_4$.   The resulting precipitate was collected on a filter, washed with 0.7 saturated $(NH_4)_2SO_4$, drained free of liquid, and then dried in a vacuum desiccator.   The light yellow product weighed about 100 mg.

Solanain—This enzyme was prepared from the fruit of *Solanum elaeagnifolium*.[4]   Bodansky's isolation procedure (2) was modified as follows:

The fresh fruit was ground and extracted with dilute phosphate buffer (pH 7.5), and the extract centrifuged free of solids.   The dark green solution was made up to about 0.7 saturation with solid $(NH_4)_2SO_4$.   The resulting precipitate of crude enzyme was centrifuged down, collected on a Buchner filter, washed with 0.7 saturated $(NH_4)_2SO_4$, and drained dry.   The precipitate was

[2] Pineapple fruit was kindly sent to us by Dr. J. L. Collins of the Pineapple Producers Experiment Station of the University of Hawaii.

[3] This milkweed, which was found growing wild near Pinole, California, differs greatly from *Asclepias speciosa* in size and structure.   It is a small weed, usually growing to less than a foot in height, and it yields but a drop or two of latex per plant.

[4] Collections of the fruit were kindly sent to us by Dr. R. Chandler of the Botany Department, and Dr. W. H. Brown of the Zoology Department, of the University of Arizona.

readily dissolved in water, and the enzyme reprecipitated by adding 4 volumes of acetone. The precipitate was washed with acetone and drained free of liquid. It was again dissolved in water, precipitated by 0.7 saturation with $(NH_4)_2SO_4$, and collected on a filter. Finally, the enzyme preparation was precipitated twice more from aqueous solution by 4 volumes of acetone, washed with acetone and ether, and dried in a vacuum desiccator. About 0.8 gm. of white product was the yield per 100 gm. of fresh fruit.

Asclepain s and Papain—These enzymes were prepared as previously described (3).

Methods

The activating or inhibiting effect of different reagents on the proteolytic activity of the enzymes was measured by the same technique used in previous studies with asclepain s. The enzyme solutions were treated with the specified reagents for an hour at room temperature in the cases of asclepain m and bromelin, and for about 12 hours in the experiments with solanain. Then the proteolytic activity of the treated solutions was measured on 2 per cent Van Slyke casein at 40° by the Northrop and Kunitz non-protein nitrogen method (4). In the measurements with asclepain m and bromelin, the casein substrate, buffered at pH 7.5, was digested for 30 minutes. For solanain the substrate was buffered at pH 8.5, and the digestion time was 60 minutes. The original enzyme solutions contained 1.5 mg. of asclepain m, and 3.0 mg. of bromelin or solanain, per ml.

The degree of activation or inhibition is expressed in each case as the ratio of the activity of the treated enzyme to that of the untreated enzyme solution. The activity values, expressed as milliequivalents of non-protein nitrogen produced in 6 ml. of digestion mixture in a definite time, are recorded for the untreated enzymes, but are omitted for all the treated enzyme solutions, and only the ratios are given. Enzyme solutions, when treated with maleic acid, always stood at least 12 hours before further treatment.

In certain special experiments, reported in Table III and elsewhere in the text, proteolytic activity was measured by Anson's hemoglobin method (5).

Asclepain m and Bromelin—The results of the activation-inhibition measurements with asclepain m and bromelin are given in Tables I and II. The behavior of these enzymes, in most

TABLE I

Activation-Inhibition of Asclepain m

The untreated enzyme solution, diluted with 2 volumes of water, produced 0.172 to 0.178 milliequivalent of non-protein nitrogen in 6 ml. of digestion mixture.

Reagent added to enzyme solution		Ratio of activity to that of untreated enzyme
Inhibitor	Activator	
None	0.05 M cysteine	2.98
"	0.1 " NaCN	2.78
"	0.1 " H_2S	2.66
0.005 M H_2O_2	None	0.02
Same	0.05 M cysteine	2.70
0.001 M $K_3Fe(CN)_6$	None	0.02
Same	0.1 M NaCN	2.74
0.0005 M I_2	None	0.06
Same	0.1 M NaCN	0.15
0.01 M iodoacetic acid	None	0.01
Same	0.05 M cysteine	0.00
0.03 M maleic acid	None	0,08
Same	0.1 M NaCN	2.65
5 mg. Cu_2O	None	0.04
Same	0.1 M H_2S	0.08
0.001 N Ag^+ or Hg^{++}	None	0.00–0.02
1×10^{-4} N Ag^+	"	0.20
3×10^{-5} " "	"	0.52
1×10^{-5} " "	"	0.82
0.01 N Ag^+	0.1 M H_2S	0.04
Same	0.1 " NaCN	1.46
0.01 N Hg^{++}	0.1 " H_2S	0.48
0.001 " Ag^+	0.1 " "	2.00
Same	0.1 " NaCN	2.12

respects, resembles the effects that have been reported for papain and asclepain s.

It has been pointed out by Anson (6) and by Hellerman (7) that sulfhydryl groups with different degrees of reactivity may be present in different native proteins, and sometimes in a single

protein. Balls and Lineweaver (8) likewise conclude that the —SH of native crystalline papain is able to react with certain

TABLE II

Activation-Inhibition of Bromelin

The untreated enzyme solution, diluted with 2 volumes of water, produced 0.223 to 0.255 milliequivalent of non-protein nitrogen in 6 ml. of digestion mixture.

Reagent added to enzyme solution		Ratio of activity to that of untreated enzyme
Inhibitor	Activator	
None	0.06 M cysteine	2.28
"	0.03 " "	2.04–2.16
"	0.1 " NaCN	2.15
"	0.1 " H_2S	1.18–1.25
"	0.5 " Na_2S	1.10
0.003 M H_2O_2	None	0.04
Same	0.1 M NaCN	0.84
0.01 M $K_3Fe(CN)_6$*	None	0.60
Same	0.1 M NaCN	1.90
0.001 M $KMnO_4$	None	0.16
Same	0.1 M NaCN	1.18
0.001 M I_2	None	0.00
Same	0.03 M cysteine	0.26
0.02 M iodoacetic acid	None	0.01
Same	0.1 M NaCN	0.00
0.03 M maleic acid	None	1.08
Same	0.1 M NaCN	2.18
5 mg. Cu_2O	None	0.08
Same	0.1 M H_2S	0.18
0.01 N Ag^+ or Hg^{++}	None	0.00–0.02
1×10^{-4} N Ag^+	"	0.22
2×10^{-5} " "	"	0.62
0.67×10^{-5} N Ag^+	"	0.82
0.01 N Ag^+	0.1 M H_2S	0.01–0.04
Same	0.5 " Na_2S	0.50
"	0.1 " NaCN	1.76–1.90
0.001 N Ag^+ or Hg^{++}	0.1 " H_2S	1.26–1.28
0.001 " "	0.1 " NaCN	2.12

* This reagent was allowed to act for 12 hours.

reagents but not with others. If it is assumed that reversibly oxidized thiol groups in enzymes can likewise have different re-

activities, one has a further basis for interpreting activation-inhibition differences.   The responses of the enzymes depend not only upon the oxidation-reduction potentials of the reagents, but upon the particular conditions that determine the reaction rates (9).

Activation by Reducing Agents—In Table I it is seen that asclepain m is activated to approximately the same degree by different reducing agents if sufficiently high concentrations are employed. This resembles the finding of Balls and Lineweaver (1) with crystalline papain.   It seems likely that cysteine, cyanide, and H_2S all cause the same chemical change.   To account for this on the basis of the formation of thiol groups in the enzymes, it must be assumed that CN^- is oxidized to CNO^- and H_2S to S.

In the case of bromelin (Table II), cysteine and cyanide activate to about the same degree, while H_2S and Na_2S produce a much lower degree of activation.   Rather than postulate a different reaction product in the latter case, as was tentatively done by Hellerman and Perkins (10) for crude papain, it may be simply that the oxidized sulfhydryl groups of bromelin are not readily reducible by sulfides.

Fruton and Bergmann (11) found that papain, activated with cysteine or cyanide, becomes completely inactive to synthetic peptides when precipitated by isopropyl alcohol, and that the enzyme precipitate recovers full activity when again treated with the activators.   This led them to favor the older view that these activators form dissociable compounds with papain, and therefore function as coenzymes rather than as reducing agents.

Experiments like those of Fruton and Bergmann were carried out by us on papain and asclepain s.   The details are, briefly, as follows:

Preparations of asclepain s and papain were dissolved in 0.05 M neutral cysteine or cyanide solutions.   The enzymes were precipitated with 4 volumes of ethyl or isopropyl alcohol, collected on Buchner filters, and washed with absolute alcohol.   From the precipitates, solutions were made up that contained equal concentrations (0.2 mg. per ml.) of each enzyme in both pure water and in 0.05 M HCN (pH 7).   The proteolytic activity of these solutions was then measured by Anson's hemoglobin method, with 10 minute digestions at 30°.

The results in Table III show that asclepain *s*, activated with cysteine or HCN, loses only 10 per cent of its activity in the above precipitation procedure. Activated papain loses about half its activity when precipitated by ethyl or isopropyl alcohol. When the papain precipitate was strongly aerated, there was a large irreversible loss of activity.

In the opinion of the authors, the reversible losses in activity are probably produced by mild oxidation of the enzymes by oxygen, owing to the presence of catalyzing impurities, and not to dissociation of an enzyme-activator compound. The energetic aeration of the papain precipitate probably oxidized the active

TABLE III

Effect of Alcohol Precipitation on Activity of Asclepain s and Papain

Enzyme	Initial activator	Alcohol used to ppt. enzyme	Proteolytic activity of redissolved enzyme (tyrosine in 6 mL. digestion mixture)	
			Non-activated enzyme	Enzyme treated with HCN
			m.eq. $\times 10^3$	*m.eq.* $\times 10^3$
Asclepain *s*	Cysteine	Ethyl	6.7	7.3
	HCN	"	6.4	7.2
Papain	Cysteine	"	3.0	6.6
	"	Isopropyl	3.2	7.1
	"	Ethyl, ppt. aerated 30 min. on filter	0.45	1.3

sulfhydryl groups beyond the reversible stage. It is significant that highly purified papain is stable toward oxygen, and can be salted-out of 70 per cent alcohol by lithium sulfate, and subsequently recrystallized from water without loss of activity (1). This evidence opposes the view that alcohol dissociates a papain-activator complex.

Reversible Inactivation by Oxidants—When asclepain *m* is completely inactivated by dilute H_2O_2 or ferricyanide, 90 to 100 per cent of the full activity can be subsequently restored by the addition of excess cyanide or cysteine. These effects strongly suggest an oxidation-reduction process. An explanation in terms of the dissociable complex theory seems less plausible.

When bromelin is inactivated by dilute H_2O_2 or $KMnO_4$, subsequent treatment with cyanide does not produce full activation. This suggests that part of the sulfur is oxidized beyond the reversible stage.   The partial inactivation obtained with ferricyanide (acting for a much longer time) and the subsequent reactivation closely resemble the effects obtained by Hellerman and Perkins with impure papain (10).   Apparently, ferricyanide is unable to oxidize all of the sulfhydryl, but, like H_2O_2 and $KMnO_4$, can oxidize part of it past the reversible stage.   It may be noted in this connection that the "free" sulfhydryl groups of native egg albumin do not react at all with ferricyanide (6), even at pH 9.6.

Ketene—The irreversible inactivations found with iodine suggest that aromatic groups may be iodinated in these reactions.   In connection with this possibility, it was of interest to test the action of ketene on papain.[5]   This reagent inactivates pepsin presumably by acetylating the phenolic hydroxyl groups in the enzyme (12). The course of the inactivation of papain by ketene was found to resemble that of pepsin.   Ketene, acting on a 0.1 per cent solution of papain (activated with HCN) at 0°, and buffered at pH 5.5, caused a 20 per cent loss in activity in 5 minutes, and a 70 per cent loss in 1 hour.

Since it seemed possible that ketene might acetylate the —SH groups of papain, this was guarded against in another experiment by mildly oxidizing the enzyme with dilute H_2O_2 before subjecting it to acetylation.   In this experiment the papain lost 55 per cent of its potential activity in 5 minutes, and 95 per cent in 1 hour, upon treatment with ketene.

These results suggest that ketene may react with groups in papain other than sulfhydryl, which are also essential for proteolytic activity.   The slow rate of the reaction makes it seem unlikely that these are primary amino groups.   Balls and Lineweaver (1) likewise believe that the NH_2 groups of papain are unrelated to the enzyme activity.   Accordingly, in line with the deductions for pepsin, it seems possible that phenolic groups, in addition to —SH, are essential for the proteolytic activity of papain.

Iodoacetic Acid—This reagent, which reacts vigorously with

—SH compounds, completely inactivates asclepain m and bromelin. None of the activity is restored by adding an excess of activator. Balls and Lineweaver (8) have shown that the inhibition of papain activity is produced by 1 molecular equivalent of iodoacetate, and have detected hydriodic acid as a product of the reaction, so that the reaction mechanism seems fairly well established.

According to Maschmann (13), the inactivation of papain by iodoacetate is reversed by precipitation of the enzyme with alcohol. This experiment was repeated by us, using 0.02 M iodoacetic acid (pH 7) completely to inactivate a 0.3 per cent papain solution. Following alcohol precipitation, we found no recovery in the proteolytic activity of the enzyme.

Cuprous Oxide—This substance, which reversibly inactivates papain at pH 5 (10), inactivates asclepain m and bromelin irreversibly at pH 7. The nature of the reaction here is not known.

Maleic Acid—Morgan and Friedmann (14) found that the incubation of papain with maleic acid at 37° and at pH 4.7 produced 70 per cent inhibition of the enzyme after 16 hours. By analogy with the reactions of thiol compounds, these workers conclude that maleic acid probably forms an addition compound with reduced papain. No attempt was made to reverse the inactivation.

We have obtained 90 per cent inhibition of papain with maleic acid at pH 7.0 by incubation for 5 hours at 37°. This inactivation was about 70 per cent reversed by the subsequent addition of excess cyanide.[6] In Table I it is seen that asclepain m is completely inhibited by maleic acid, and is fully reactivated by cyanide. Asclepain s behaves in the same manner (data not recorded).

A plausible explanation of the reversal in these cases is that maleic acid inactivates by oxidizing the enzymes, and that it is itself reduced to the rather stable substance, succinic acid; then cyanide reactivates by reducing the oxidized forms of the enzymes.

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[6] The same conditions were employed as in measurements with asclepain m and bromelin, except that the hemoglobin method was used to measure proteolytic activity. Following the inactivation by 0.01 M maleic acid, the papain solution (containing 0.6 mg. of enzyme per ml.) was activated to 68 per cent of the full activity value for the cyanide-treated enzyme.

The —SH of bromelin apparently does not react readily with maleic acid, as its activity is not appreciably altered by this reagent.   When excess cyanide is also added to the solution, the enzyme becomes fully activated.

Heavy Metal Ions—Hellerman (9) explains the inactivation of papain by heavy metal ions as being due to the formation of mercaptides.   Activators, such as HCN and H_2S, remove the metal and liberate the —SH groups of the enzyme.

When asclepain m and bromelin are inactivated by adding 0.001 N Ag^+ or Hg^{++} to the enzyme solutions, it is possible, by the subsequent addition of excess sulfide or cyanide, to activate asclepain m to 75 per cent, and bromelin to 100 per cent, of the full latent activity.   But when 0.01 N metal ions are added, excess sulfide produces 0, or only slight activation, while excess cyanide reactivates asclepain m about 50 per cent, and bromelin about 85 per cent.   This depressed reactivation, which results when relatively concentrated solutions of Ag^+ or Hg^{++} are used, is most marked with asclepain s.   The activation of this enzyme by 0.001 N Ag^+ is completely reversed by 0.1 M H_2S (data not recorded).   But if 0.01 N Ag^+ is used, H_2S restores none of the proteolytic activity (3).   The reason for this irreversibility is not known.

By use of different concentrations of very dilute silver ion, the enzymes can be inactivated to varying degrees.   It is interesting to compare the quantities of Ag^+ which cause 50 per cent inactivation of a given amount of the different enzyme preparations. These quantities, calculated from the data in Tables I and II, are 2.0×10^{-5} milliequivalent of Ag^+ per mg. of asclepain m, and 2.5×10^{-5} milliequivalent of Ag^+ per mg. of bromelin.   From other published data, the corresponding values for asclepain s (3) and papain (15) were calculated to be 0.8×10^{-5} and 1.1×10^{-5} milliequivalent of Ag^+ per mg. of enzyme, respectively.

Solanain—The results of activation-inhibition experiments with solanain are given in Table IV.   It is seen that the behavior of this protease is very different from that of the previous enzymes. Reducing agents, such as cysteine, cyanide, and H_2S, have no significant effect on the activity of solanain.   Relatively concentrated solutions of oxidants such as hydrogen peroxide and ferricyanide fail to inactivate the enzyme.   Iodine in neutral solution

does inactivate solanain, but it appears unlikely that the reaction is one of oxidation. Iodoacetic acid, maleic acid, and cuprous oxide, which react with sulfhydryl compounds, and which commonly inactivate papainases, have no effect on the activity of solanain. Phenylhydrazine, which activates papain (16) and asclepain s (3), is likewise without effect.

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TABLE IV

Activation-Inhibition of Solanain

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The untreated enzyme solution, diluted with 2 volumes of water, produced 0.275 to 0.292 milliequivalent of non-protein nitrogen in 6 ml. of digestion mixture.

Reagent added to enzyme solution		Ratio of activity to that of untreated enzyme
Inhibitor	Activator	
None	0.04 M cysteine	1.6 –1.12
"	0.1–0.5 M NaCN	1.10–1.06
"	0.1 M H_2S	1.06
0.038 M H_2O_2	None	0.96
0.02 " $K_3Fe(CN)_6$	"	0.98
0.01 " I_2	"	0.07
Same	0.1 M NaCN	0.13
0.03 M iodoacetic acid	None	0.98
0.03 " maleic acid	"	0.98
5 mg. Cu_2O	"	1.00
0.02 M phenylhydrazine	"	1.03
0.2 " $NaNO_2$, pH 3.8	"	0.50
Acetate buffer, pH 3.8	"	0.94
0.01 N Ag^+ or Hg^{++}	"	0.01–0.06
0.002 " "	"	0.14
0.001 " " or Hg^{++}	"	0.30–0.34
0.0004 N Ag^+	"	0.56
0.01 N Ag^+ or Hg^{++}	0.1 M H_2S or 0.1 M NaCN	0.00–0.13

Philpot and Small (17) have shown that nitrous acid acts on pepsin to produce a yellow *diazo compound* which has 50 per cent of the original activity. It is interesting to note that HNO_2 (liberated from $NaNO_2$ under comparable conditions) likewise acts on solanain to form a yellow product which has about half the original activity. While the nature of the reaction is not known in the case of solanain, it is possible that phenol groups also are involved as in the case of crystalline pepsin. If this is true, the

irreversible inactivation of solanain by iodine could be due to the iodination of phenol groups in the enzyme, similar to the reaction of pepsin (18).   Further evidence for this theory is the fact that ketene (at 0° and pH 5.5) produces 50 per cent inactivation in 5 minutes and complete inactivation of solanain within an hour.

Solanain is inactivated by 0.01 N Ag^+ or Hg^{++}.   As with the previous enzymes, the inactivation by these relatively concentrated solutions is irreversible.   By partial inactivations with more dilute Ag^+ solutions, it is found that 1.6×10^{-4} milliequivalent of Ag^+ causes 50 per cent inactivation of 1 mg. of solanain.   This quantity of Ag^+ is about 10 times as great as the corresponding amounts listed for asclepain m, asclepain s, papain, and bromelin. If it is assumed that the impurities in each enzyme have an approximately equal effect, the higher value for solanain again suggests that this enzyme has a different active group than the other proteases.[7]

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SUMMARY

1. A study was made of the activation-inhibition reactions of three partly purified plant proteases, bromelin of pineapple, asclepain m of the milkweed, *Asclepias mexicana*, and solanain of the horse-nettle, *Solanum elaeagnifolium*.

2. The reactions of bromelin and asclepain m resemble those of papain and asclepain s (protease of *Asclepias speciosa*), and are indicative of the presence of sulfhydryl as a group essential to the activity of these enzymes.

3. Solanain is not affected by oxidizing or reducing agents, or by reagents which react with —SH groups.   This enzyme is, therefore, not a papainase.

4. The inactivations produced by nitrous acid and ketene indicate that phenolic groups may be essential for the activity of solanain.   The course of the inactivation of papain by ketene is

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[7] It may be of interest that solanain resembles trypsin and chymotrypsin, but differs from all papainases, in that it does not readily turn casein white during the digestion process.

favorable to the view that phenolic as well as —SH groups are necessary for the activity of this enzyme.

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PLANT PROTEASES

II. pH-ACTIVITY CURVES

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In continuation of the authors' comparative studies on plant proteases (1, 2), this paper describes the pH-activity curves of a number of these enzymes. The proteases investigated were papain, bromelin, solanain, asclepain m, and asclepain s. Solanain is obtained from the berry-like fruit of the horse-nettle, *Solanum elaeagnifolium*. The last two enzymes are from the latex of two different milkweeds, *Asclepias mexicana* and *Asclepias speciosa*.

Northrop (3) has shown that the pH-activity curves of the digestion of hemoglobin and casein by pepsin and trypsin parallel the titration curves of the proteins. Accordingly, the rate of proteolysis is proportional to the degree of dissociation of the substrate. The digestion of several native proteins by the papainases, asclepain s (1), was found to follow this relationship approximately, but when the same proteins were denatured in urea solution, the digestion curves were all very much alike, despite the different isoelectric points of the proteins, and the optima were all near pH 7.0.

Until very recently it was generally accepted that papain digested only the neutral form of the proteins, and as a consequence, exhibited the greatest activity at the isoelectric pH of the substrate. In the present paper, the character of the pH-activity curves of plant proteases is shown to depend more upon the electrochemical nature of the enzymes than upon the degree of dissociation of the substrates. The substrates are hemoglobin and ovalbumin denatured in urea. Native hemoglobin cannot be used, since it is very poorly digested by proteases. Pure, recrystallized, native

ovalbumin is even more indigestible.[1] None of the plant pro-
teases tested digested this substrate appreciably.

Materials and Methods

Proteases—The methods of preparing the different proteases
have been previously described (1, 2). The enzyme solutions
used in the activity measurements were prepared by dissolving
specified weights of the dry preparations in about 0.1 M NaCN sol-
ution (titrated to pH 7), and then diluting the solution 10-fold
with water, so that the cyanide concentration became 0.01 M.

Protein Substrates. Hemoglobin—3 ml. portions of 32 per
cent hemoglobin, prepared by the method of Anson (4), were
added to each of a number of beakers containing 20 gm. of urea
and 20 ml. of 0.25 N NaOH. Then each solution was adjusted
to a different pH value by adding 1 N acetic, phosphoric, or boric
acid, the glass electrode being used to measure the pH. The so-
lutions were lastly made up to 50 ml. volume by adding water.
The final protein concentrations were 2 per cent, and the urea
6.6 M in every case.

Ovalbumin—Crystalline ovalbumin was prepared by the method
of Sörensen (5). The product was recrystallized twice. The same
technique as before was used in preparing solutions of this protein,
except that 5 ml. of 20 per cent ovalbumin were added to the al-
kaline urea solution. The final solutions again contained 2 per
cent protein in 6.6 M urea.

Activity Measurements—The pH-activity curves of each protease
on hemoglobin and ovalbumin were obtained by digesting 5 ml.
portions of the protein substrates (buffered at various pH values)
with 1 ml. volumes of enzyme solution. The hemoglobin solutions
were digested 20 minutes, and the ovalbumin solutions 15 minutes,
at a temperature of 25°. The amount of digestion was measured
by Anson's hemoglobin method (4). The intensity of the blue
color produced with the phenol reagent was determined with an
Evelyn photoelectric colorimeter (6). The latter was calibrated
with a series of standard tyrosine solutions. The proteolytic ac-

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[1] In a previous paper (1), we reported that Merck's egg albumin, puri-
fied somewhat by dialysis, was fairly digestible. This digestibility was
most likely due to the presence of a considerable proportion of denatured
material in the protein.

tivity is expressed as milliequivalents of tyrosine produced in 6 ml. of digestion mixture in the above specified times.

The pH-activity curves of four different proteases on denatured hemoglobin are given in Fig. 1. Bromelin is seen to have a flat optimum region at about pH 6 to 7. The curves of asclepain m and asclepain s are much alike, with optima at pH 7.5. Solanain, which has the lowest activity on hemoglobin, does not digest this
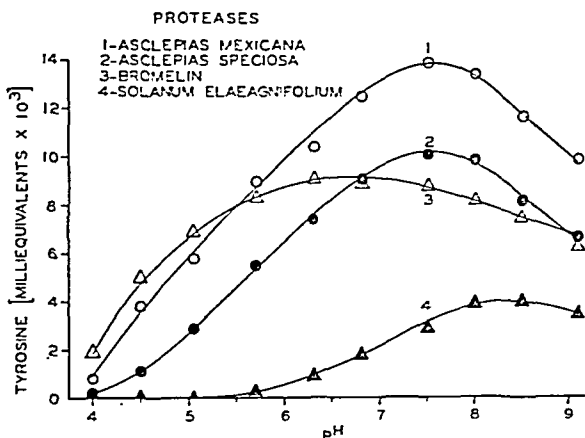
FIG. 1. pH-activity curves of plant proteases on denatured hemoglobin. The quantity of enzyme in 6 ml. of digestion mixture was 0.33 mg. in the cases of asclepain m (Curve 1) and asclepain s (Curve 2), and 0.5 mg. in digestions with bromelin (Curve 3) and solanain (Curve 4).

protein at all below pH 6. The optimum digestion rate with this enzyme occurs near pH 8.5.

Anson has found that the optimum pH of papain on hemoglobin is in slightly alkaline solution, and Lineweaver finds a similar optimum with crystalline papain.[2]

Fig. 2 gives the pH-activity curves of the same enzymes, and also that of papain, on denatured ovalbumin. The curve for bromelin has a peak near pH 7.0, while the other enzymes have optima near pH 7.5.

[2] Private communication.

Lineweaver and Hoover,[2] in agreement with our results, find that crystalline papain digests ovalbumin much more rapidly near pH 7 than at pH 4.5.   They find an optimum pH of 6.5 to 7.0 for the digestion of casein.

The fall in the digestion rates, particularly on the acid side of the pH optima, is not due to the destruction of the enzymes by the prevailing acidity or alkalinity of the digestion mixtures.   This was ascertained by comparing the activities of the enzymes after they had stood in different buffer solutions for 20 minutes at 25°. . It was found that none of the enzymes was appreciably inactivated
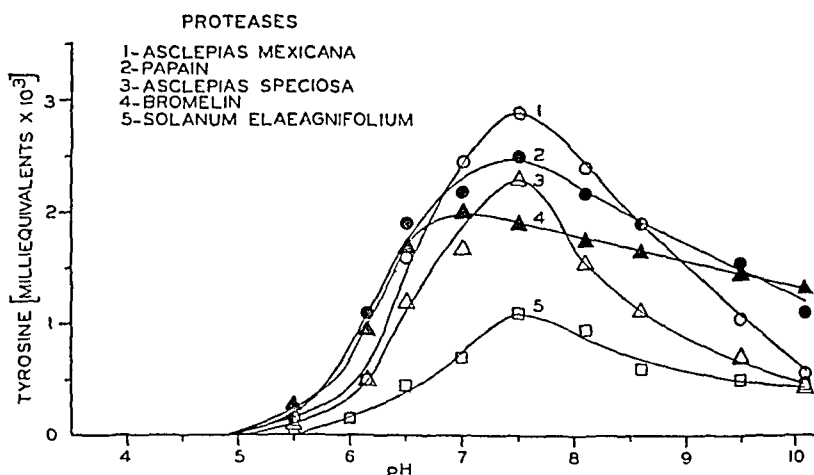
FIG. 2. pH-activity curves of plant proteases on denatured ovalbumin. The quantity of enzyme in 6 ml. of digestion mixture was 0.5 mg. in the cases of asclepain m (Curve 1), papain (Curve 2), and asclepain s (Curve 3), and 1.0 mg. in digestions with bromelin (Curve 4) and solanain (Curve 5).

in the region of pH 4 to 8.5.   At pH 9.4, asclepain m lost 10 per cent, and asclepain s 35 per cent, of its activity.   At pH 10.0, solanain was unaffected, while bromelin lost 5 per cent, asclepain m 25 per cent, and asclepain s 35 per cent of its activity, respectively.   If the pH-activity curves of the last two enzymes on ovalbumin are corrected for the above losses, the slopes on the alkaline sides more nearly approximate those of the other enzymes.

Broadly speaking, there are no marked differences in the pH optima of the hemoglobin and the ovalbumin curves.   If it is assumed that the dissociation curves of hemoglobin and oval-

bumin in aqueous solution are shifted in the same direction and to an approximately equal extent in 6.6 M urea (7), it follows that the proteins are ionized to very different degrees at the same pH values. But, since the pH-activity curves with the two proteins are rather similar, it seems unlikely that the rate of digestion in urea is closely related to the degree of ionization of the substrates.

On the other hand, there may be considerable differences in the curves of two different proteases on the same protein substrate. The curves of bromelin and solanain on hemoglobin (Fig. 1) are examples. At pH 6.0, the digestion rate with the first enzyme is near its maximum, while that with solanain is almost 0. Accordingly, it seems likely that either the degree of ionization or the configuration of the enzyme molecule is the decisive factor governing the rate of digestion of proteins in urea.

Favoring this view is the fact that papain and asclepain s, which appear to have the same optimum pH on hemoglobin and ovalbumin, both have isoelectric points in the alkaline region. The isoelectric point of crystalline papain is near pH 9.0 (8), while that that of asclepain s is on the alkaline side of pH 7.6. The latter fact was ascertained by the authors by electrophoretic measurements. In the Tiselius apparatus, at pH 7.6 and 1.5°, both the protein nitrogen and proteolytic activity of asclepain s migrated rapidly toward the cathode.[3]

SUMMARY

1. pH-activity curves were determined for several plant proteases on denatured hemoglobin and ovalbumin in concentrated urea solution.

2. The optimum pH of each protease on ovalbumin was between 7 and 7.5.

3. With hemoglobin, the optimum pH of the proteases ranged from 6.5 to 8.5.

4. The digestion rates at different pH values appeared to de-

[3] We thank Dr. C. H. Li of the Institute of Experimental Biology for the use of his electrophoresis apparatus.

pend more upon the nature and charge of the enzyme than upon the sign of the electrical charge, or upon the degree of dissociation of the substrate.

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PLANT PROTEASES

III. KINETIC PROPERTIES

By DAVID M. GREENBERG AND THEODORE WINNICK

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In the previous papers of this series, the activation-inhibition reactions (1) and pH-activity curves (2) of several different plant proteases were described.

The present paper, which is a continuation of earlier studies (3), deals with the kinetic aspects of the reactions of these enzymes. It is shown that information on properties such as rates of heat inactivation, and the nature of the enzyme-substrate intermediary compounds, is of value for characterizing and comparing different proteases. The enzymes reported upon here are papain, bromelin, asclepain *s*, asclepain *m*, and solanain.

From a practical standpoint, a knowledge of the degree of heat stability of enzymes at different temperatures is of considerable use when one desires to produce the maximum enzymic action in a relatively short time. For example, it was found (3) that papain is fairly stable at 65–70°, whereas bromelin is rapidly destroyed at these temperatures. This indicates the advantage in using the former protease when rapid digestion at higher temperatures is desired.[1]

Materials and Methods

Enzymes—The methods of preparing the different proteases have already been described (1, 3). The protease solutions used in the various measurements were prepared by dissolving specified weights of dry enzymes in 0.05 M NaCN solution, buffered to pH 7.5.

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[1] Cyanide-activated papain and urea in 10 per cent concentration, acting at a temperature of 50–60°, have been employed by one of us (D. M. G.) for rapid digestion of the protein material from rat carcasses.

Method of Measuring Rates of Heat Inactivation—The technique used in making these measurements has been previously given (3). The residual proteolytic activities of the enzyme solutions (which were heated at a definite temperature for different lengths of time) were measured by Anson's hemoglobin method (4), with 15 minute digestions at 30°.   The substrate was 2 per cent hemoglobin in 6.6 M urea, buffered at pH 7.5.[2]

Method Used for Study of Enzyme-Substrate Compounds—A graded series of substrates, containing varying concentrations of hemoglobin, was prepared by quantitively diluting samples of a relatively concentrated solution of hemoglobin in 6.6 M urea at pH 7.5[2] to different degrees with 6.6 M urea solution, likewise buffered to pH 7.5.   The activity of a given enzyme solution was determined by digesting 5 ml. portions of each substrate for exactly 15 minutes at 30° by 1 ml. aliquots of enzyme solution.[3]

<div align="center">EXPERIMENTAL</div>

Enzyme-Substrate Intermediates—Lineweaver and Burk (5) have developed graphic methods for determining the nature of enzyme-substrate intermediates and evaluating the dissociation constants of these compounds.   According to their equations, which are based on the Michaelis-Menten theory, the plot of the ratio of initial substrate concentration to reaction rate (S/V) against initial substrate concentration (S) yields a straight line for the case in which the enzyme-substrate compound consists of 1 molecule each of enzyme and substrate.[4]

In Fig. 1, S/V is plotted against S for the several different proteases.   It is seen that the experimental points fall fairly well along straight lines in each case.   This linear relation indicates that the intermediate compound which initiates the hydrolysis of hemoglobin in urea solution consists in all cases of 1 molecule each of hemoglobin and enzyme.

[2] pH 8.5 was used for the measurements with solanain.

[3] The temperature was 40° in digestions with solanain.

[4] Dr. Lineweaver has informed us that, according to mathematical analysis, this method of plotting involves less error than plots of $1/V$ against $1/S$.   This confirms our empirical observations, which indicated that the experimental points were more widely scattered when plotted by the latter method, particularly for lower values of S, where the error in measuring V is greater.
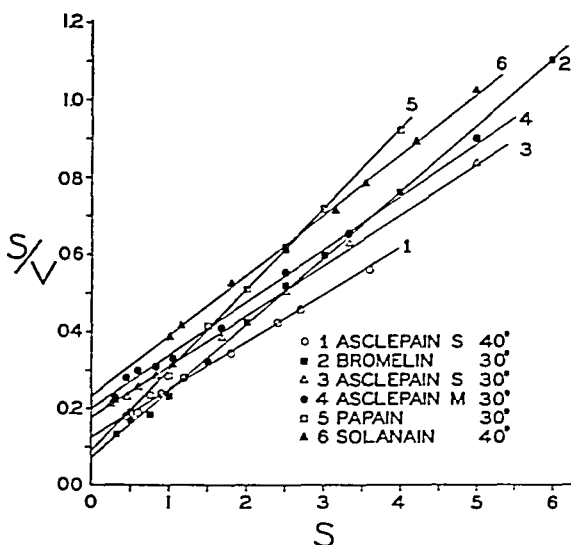
FIG. 1. Nature of the enzyme-substrate intermediates of plant proteases. S is the initial concentration (in per cent) of protein in the substrate, and V is the digestion rate, expressed as milliequivalents of tyrosine $\times 10^3$, produced in 6 ml. of digestion mixture in 15 minutes. In the case of solanain (Curve 6), S is per cent protein $\times 10$. Curve 1 is reproduced from a previous paper (3) for purposes of comparison. It represents 5 minute digestions by 0.25 mg. of asclepain s. For the other curves, the mg. of enzyme per 6 ml. of digestion mixture are as follows: Curve 2, 0.33; Curve 3, 0.20; Curve 4, 0.167; Curve 5, 0.20; and Curve 6, 0.50.

TABLE I

Enzyme-Substrate Dissociation Constants

Protease	Temperature	K_s
	°C.	
Asclepain m	30	1.45
" s	30	1.35
	40	1.05
Papain	30	0.42
Bromelin	30	0.41
Solanain	40	0.145

From the plots in Fig. 1, the value of the enzyme-substrate dissociation constant, *i.e.* the Michaelis constant, K_s, was calculated for each enzyme. These constants are given in Table I.

It is seen that asclepain m and asclepain s have practically equal K_s values at 30°, and that K_s for asclepain s does not change greatly over a 10° interval.   Papain and bromelin have lower K_s values, while the constant for solanain (at 40°) is smallest of all.

There is an interesting difference between the kinetic behavior of solanain and that of the other proteases.   With the other enzymes the initial rate of digestion continues to increase up to rather high values of the substrate concentrations.   In the case of solanain, it was found that the quantity of digestion produced during the first 15 minutes was almost constant over the range of 0.5 to 6.0 per cent initial hemoglobin concentration.[5]   This behavior of solanain largely explains the relatively low K_s value of the enzyme.

It is interesting that in practically all the types of enzymes which have been studied, the enzyme-substrate intermediate appears to consist of 1 molecule each of enzyme and substrate.   This relationship in the apparent composition cannot be taken too literally.   The similar kinetic behavior of the different enzymes, however, suggests that there is probably a basic reaction mechanism common to all.

Rates of Heat Inactivation—It has been shown that the heat inactivation of papain and bromelin follows the course of a first order reaction at certain temperatures (3).   On the other hand, the destruction of asclepain s is apparently a second order reaction (3).

It is interesting that at pH 7 heat destroys asclepain m and solanain, like papain and bromelin, in accordance with the first order equation.   The test of this is shown in Fig. 2, where the plots of log of activity (tyrosine color produced) against time of heating give straight lines.

The velocity constants of the inactivation reactions are given in Table II.   These values were calculated from the plots in Fig. 2 with the aid of the first order equation.   The half life periods of the enzymes are also recorded.   From the velocity constants, the

[5] It was found that the rates of digestion were largely independent of the initial hemoglobin concentration only during about the first 15 minutes, and that for longer times the total quantity of digestion varied with the initial substrate concentration.
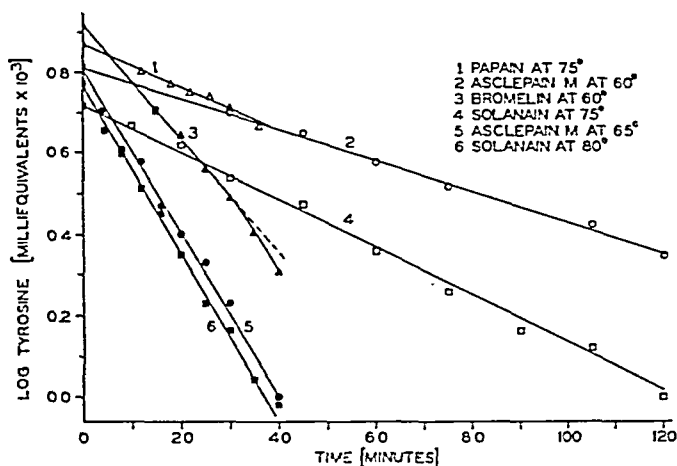
FIG. 2. Rates of heat inactivation of plant proteases. The activity is expressed in milliequivalents of tyrosine × 10³ produced in 6 ml. of digestion mixture in 15 minutes. Curves 1 and 3 are reproduced from a previous paper (3) for purposes of comparison. The concentration of enzyme in mg. per ml. was 0.16 for asclepain *m* (Curves 2 and 5), and 1.0 for solanain (Curves 4 and 6).

TABLE II

Comparison of Thermal Properties of Plant Proteases

Enzyme	Temperature	Velocity constant	Half life period	Critical thermal increment
	°C.	K × 100	min.	calories per mole
Papain	75	1.25	56	
	80	6.0	11.5	75,000
Solanain	75	1.35	51	
	80	4.8	14.5	62,000
Asclepain *m*	60	0.88	78	
	65	4.8	14.5	76,000
Bromelin	60	3.2	21.5	
	65	8.8	7.9	46,000
Asclepain *s*	60	0.95	13	
	65	1.65	6.5	25,000

critical thermal increments (energies of inactivation) are calculated, with the van't Hoff-Arrhenius equation

It is seen that papain and solanain are destroyed at approxi-

mately equal rates at 75° and 80°,[6] and that the former enzyme has a somewhat higher critical thermal increment. The other proteases are all much less resistant to heat inactivation; this is apparent from the fact that there is about a 15° temperature differential between the rates of inactivation of these enzymes and the same rates for papain or solanain.

The results suggest that the magnitude of the critical thermal increment is not related to the degree of thermal lability of the enzyme.   For example, the increments for asclepain m and papain are equal, although the latter enzyme is far more resistant to heat inactivation.

The critical increments of the above enzymes resemble the values which have been found for animal proteases, and this suggests that the reactions are similar in the denaturation of either plant or animal proteases.

It is seen that asclepain m and asclepain s appear to differ markedly in the kinetics of their inactivation, although their heat stabilities are approximately equal.   It is possible that the low thermal increment of asclepain s is related to its second order inactivation rate.   The irreversible inactivation of trypsin also follows a second order reaction between pH 2 and 9 (7).   To explain this, it has been suggested that the native (active) trypsin hydrolyzes the denatured form with which it is in equilibrium.   A similar situation seems possible in the case of asclepain s.

It is noteworthy that the activation-inhibition reactions, pH-activity curves, and enzyme-substrate dissociation constants of asclepain m and asclepain s are almost identical.   If the differences in the kinetics of heat inactivation can be attributed to the presence of different impurities, it would appear quite probable that the two *Asclepias* proteases are chemically identical.

On the other hand, the several lines of indirect experimental evidence suggest that the molecules of papain and bromelin have somewhat different structures.   Further, it appears likely that the above enzymes, which are all papainases, differ even to a greater degree in their structural composition from such "non-papainases" as solanain.   It may be possible to decide these questions with

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[6] Our results with solanain indicate a higher degree of resistance to temperature than do the data of Bodansky (6). For example, Bodansky found that 60 per cent of the enzyme was destroyed in 15 minutes at 70°.

more certainty when the crystalline forms of the different proteases become available for study.

Technical assistance was furnished by the personnel of the Works Progress Administration, Official Project 65-1-08-62, assigned to the University of California.

SUMMARY

1. The Michaelis constants were evaluated for five different plant proteases: papain, bromelin, asclepain *m*, asclepain *s*, and solanain. It was shown that in every case the enzyme-substrate intermediary compound consisted of 1 molecule each of enzyme and protein.

2. The heat inactivation of asclepain *m* and solanain followed the course of a first order reaction. The enzymes resembled papain and bromelin in their high critical thermal increments. The magnitude of the critical thermal increments did not appear to be related to the degree of heat lability of the enzymes.

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HEAVY CARBON AS A TRACER IN BACTERIAL FIXATION OF CARBON DIOXIDE*

Sirs:

Wood and Werkman[1] postulated that succinic acid is formed by combination of CO_2 and a 3-carbon compound. The validity of this suggestion has now been examined, with C_{13} as a tracer with suspensions of bacteria in C_{13} $NaHCO_3$ solutions. The C_{13} in the products has been determined with a mass spectrograph.

The natural molecular percentage ratio $C_{13}:C_{12}$ is approximately 1.10. Succinic acid is the only product from galactose which contained a concentration of C_{13} substantially differing from that of galactose. Evidently C_{13} CO_2 was fixed in succinic

	Initial NaHCO₃	Final NaHCO₃	Products*			
			Alcohols	Volatile acids	Succinic acid	CO₂
E. coli, mμ per l..........	110.0	143.8	51.4†	20.6,‡ 1.12§	52.5	33.8
Galactose, % $C_{13}:C_{12}$......	4.36	2.80	1.07	1.08	1.49	
E. coli, mμ per l..........	149.1	167.8	0.0	87.2,‡ 70.2§	34.3	18.7
Pyruvate, % $C_{13}:C_{12}$.......	2.65	2.36		1.03, 1.53	1.27	
P. pentosaceum, mμ per l..	108.1	80.1	6.2‖	6.1,‡ 90.6¶	21.7	−28.0
Glycerol, % $C_{13}:C_{12}$.......	2.62	2.15	1.19	1.25	1.30	

* 8.63 mμ of lactic acid and 1.08 mμ of H_2 also were formed in the pyruvate fermentation; 12.00 mμ of H_2 in the galactose fermentation.

† Ethyl, ‡ acetic, § formic, ‖ propyl, ¶ propionic.

acid. Pyruvate gave similar results. Formic acid contained fixed CO_2. The quantitative distribution of C_{13} in products of *Propionibacterium pentosaceum* shows general distribution.

[1] Wood, H. G., and Werkman, C. H., *Biochem. J.*, **34**, 129 (1940).

Probably CO_2 is fixed by 3-C and 1-C addition, the 4-C compound being converted to propionic acid and propyl alcohol containing fixed CO_2. The observed dilution of C_{13} in the final $NaHCO_3$ indicates that formation of CO_2 occurred from carbon originally from glycerol.

The succinate was converted into malate and fumarate; a heart muscle preparation was used. A portion was then oxidized with permanganate.[2] Malate yields 2 molecules of CO_2 and 1 of acetaldehyde; fumarate yields CO_2 and only a trace of acetaldehyde. A second portion was converted into oxaloacetate with *Micrococcus lysodeikticus*. 1 CO_2 was liberated from each oxaloacetate, with citric acid and aniline.[3] Both methods demonstrate that substantially all the fixed CO_2 is in the carboxyl groups of succinic acid and that the methylene carbons have only the natural complement of C_{13}.

Escherichia coli and *Propionibacterium pentosaceum* fix CO_2 with formation of succinic acid. A possible mechanism is as follows: $CO_2 + CH_3COCOOH = COOH \cdot CH_2 \cdot CO \cdot COOH$, $COOH \cdot CH_2COCOOH + 4H = COOHCH_2 \cdot CH_2COOH + H_2O$.

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[2] Friedemann, T. E., and Kendall, A. I., *J. Biol. Chem.*, **82**, 23 (1929).
[3] Ostern, P., *Z. physiol. Chem.*, **218**, 160 (1933).

PURIFICATION OF THE INTERSTITIAL CELL-STIMU-LATING AND FOLLICLE-STIMULATING HORMONES OF THE PITUITARY

Sirs:

In a previous publication Jensen, Simpson, Tolksdorf, and Evans[1] described the preparation from sheep pituitary of a follicle-stimulating fraction containing 40 rat units per mg. and of an interstitial cell-stimulating fraction[2] containing 80 rat units per mg. Further purification of these two gonadotropic fractions was carried out by fractional precipitation with ammonium sulfate over a pH range of 5 to 6 and at a protein concentration of approximately 1 per cent.

The interstitial cell-stimulating principle was found to be precipitated from aqueous solution (pH 5 to 6) by ammonium sulfate within a range of 0.25 to 0.35 saturation. This precipitation was repeated several times until only a small amount of material precipitated up to 0.25 saturation and until very little material was left in the supernatant of the 0.25 to 0.35 saturated ammonium sulfate precipitate. The final precipitate was dissolved in water, dialyzed until free from inorganic salts, precipitated in 85 to 90 per cent alcohol, and the precipitate dried with absolute alcohol and ether. The minimal effective dose (1 rat unit) of the interstitial cell-stimulating fraction thus obtained, tested in hypophysectomized immature female rats for repair of the deficient interstitial tissue,[1] was found to be 5 γ.[3] Doses of 100 rat units of this preparation failed to display follicle-stimulating activity.

[1] Jensen, H., Simpson, M. E., Tolksdorf, S., and Evans, H. M., *Endocrinology*, 25, 57 (1939).

[2] This principle is now generally accepted to be identical with the luteinizing factor of the pituitary.

[3] Doses are sometimes expressed on a total nitrogen basis. Assuming that the principles are of a protein-like nature, 1 rat unit would contain less than 1 γ of total nitrogen.

The follicle-stimulating principle was found to be precipitated from aqueous solution (pH 5 to 6) by ammonium sulfate at 0.5 to 0.6 saturation. Purification was carried out in a manner similar to that of the interstitial cell-stimulating principle. The final fraction, obtained after several precipitations between 0.5 and 0.6 saturation of ammonium sulfate, gave a follicle-stimulating response in hypophysectomized immature female rats[1] at a minimal effective dose (1 rat unit) of 5 γ.[3] At 40 rat units the follicles showed beginning luteinization, the interstitial tissue remained partially deficient, and an estrous uterus was frequently observed. At 80 or 120 rat units definite formation of corpora lutea occurred, the interstitial tissue, however, showing only partial repair.

The above methods are relatively simple and permit the preparation of the two hypophyseal gonadotropic principles in a highly purified form.

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GLYCOLIC ACID OXIDASE

Sirs:

An enzyme which promotes consumption of oxygen by glycolic acid has been found in rat and rabbit livers. The livers had been minced with an embryo press, washed in water, and suspended in an equal volume of 0.1 M phosphate buffer, pH 7.4. The oxidase was not found in similar preparations of rat kidney, heart, or spleen or of rabbit kidney, brain, or pancreas. Addition of coenzymes (heated yeast extract) did not increase the activity of rat liver suspensions.

The activity was measured at 38° in Warburg manometers with alkali inset cups in the flasks. The total volume of the reaction mixture was 2 cc. The oxygen uptake of an enzyme blank was subtracted from that of the mixtures containing glycolic acid. With rat liver, the curve of oxygen consumption plotted against time shows a steep rise which is almost linear, followed by a much flatter, but also linear, portion. If this latter part of the curve, due to a secondary reaction, is extrapolated to zero time, it is seen that the major reaction is the consumption of $\frac{1}{2}$ mole of oxygen by 1 mole of glycolic acid. The result of the 2,7-dihydroxynaphthalene test[1] for the presence of glycolic acid is negative when the major reaction is over.

The oxygen uptake is slow when rat liver is used; for example, at least 3 hours are required for 0.6 mg. of glycolic acid to consume its theoretical 88 mm. of oxygen in the presence of 1.5 cc. of suspension. All preparations were active, but some more so than others. There was no change in activity when they stood in the refrigerator. Rabbit liver was about 3 times as active as rat liver, possibly because it was minced more finely by the embryo press.

The rate of oxygen uptake for the first 40 minutes was identical with 0.2, 0.6, and 1.0 mg. of glycolic acid in the presence of

[1] Feigl, F., Spot test, New York, 300 (1937).

equal amounts of rat liver; it was directly proportional to the amount of tissue for at least 90 minutes when 0.5, 1.0, and 1.5 cc of liver suspension were allowed to act on 1.0 mg. of glycolic acid. The reaction rate was increased about 25 per cent when phosphate buffer of pH 7.9 instead of pH 7.4 was used; the existence of an optimum on the alkaline side was thus indicated.

The oxidase was discovered during a critical survey of the Cohen[2] method for the determination of glutamic acid. In this method, succinic acid is formed from glutamic acid by treatment with chloramine-T followed by hydrolysis with HCl; the succinate is estimated by its oxygen uptake in the presence of heart succinoxidase. It was found that serine interfered if rat liver, instead of heart, was used as a source of succinoxidase. This was assumed to be due to the formation of glycolic acid from the serine. Confirmation was found in the positive outcome of color tests[1] and in an oxygen uptake equal in rate to that of glycolic acid.

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[2] Cohen, P. P., *Biochem. J.*, **33**, 551 (1939).

ON THE ISOLATION AND PROPERTIES OF CARBOXYLASE

Sirs:

We have obtained carboxylase from top brewers' yeast in highly purified and stable form. The method consists essentially of a series of controlled salt fractionations. The enzyme was found to be a diphosphothiamine magnesium protein. The best preparations contain 0.46 per cent diphosphothiamine and 0.13 per cent Mg. No other metal was found to be present. At this level of purity 1 mg. of the enzyme catalyzes the formation of 12,100 microliters of CO_2 per hour at 30°. 1 molecule of diphosphothiamine catalyzes the breakdown of 840 molecules of pyruvic acid per minute at 30°. The enzyme can be resolved into its component parts by a variety of procedures. For purposes of quantitative and reversible splitting the enzyme was precipitated thrice from ammoniacal $(NH_4)_2SO_4$ solution. The specific protein was thereby separated from the prosthetic group and metal. To reconstitute the original enzymic activity, three components are necessary, (1) the specific protein, (2) diphosphothiamine, and (3) some divalent cation. Diphosphothiamine cannot be replaced by thiamine, monophosphothiamine, coenzyme I, flavin-adenine dinucleotide, adenylic acid, or adenosine triphosphate. All the divalent cations yet tested, *e.g.* Mn, Mg, Fe, Ca, Cd, Zn, and Co, were active in the system though with varying efficiency. Monovalent and tervalent cations were inactive. There are good experimental grounds for the view that the metal is playing the rôle of a "cement" substance which binds the specific protein to the prosthetic group.

The conditions for the quantitative reconstitution of carboxylase from its component parts are complex. The speed and extent of recombination depend on factors such as order of mixing, relative proportions of the three components, salt concentration, temperature, etc. In high salt concentrations carboxylase is a firmly bound, conjugated protein, whereas in dilute salt solu-

tions or in alkaline ammonium sulfate solutions it dissociates almost completely.

There is evidence partly derived from ultracentrifuge measurements. that the molecular ratio of protein to diphosphothiamine is 1:1. The molecular ratio of diphosphothiamine to magnesium is 1:5.

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THE SULFHYDRYL GROUPS OF EGG ALBUMIN

Sirs:

The —SH groups of denatured egg albumin in guanidine hydro-chloride solution can be estimated by measuring how much ferricyanide, tetrathionate, or *p*-chloromercuribenzoate[1] is needed to abolish the nitroprusside test. Porphyrindin, the first reagent used to titrate protein —SH groups in guanidine hydrochloride solution,[2] is hard to prepare, unstable, and a dangerously strong oxidizing agent.

The —SH groups of denatured egg albumin in a solution of the detergent, duponol PC, can be estimated by measuring how much ferricyanide is reduced,[3, 4] how much ferricyanide is needed to abolish the nitroprusside test, or how much mercuribenzoate is needed to abolish the reduction of ferricyanide. The nitro-prusside test is carried out in guanidine solution after the protein has been precipitated and washed with trichloroacetic acid.

In the original porphyrindin titration procedure,[2] the denatured protein is allowed to stand in guanidine hydrochloride solution before the titrating agent is added. I have found, however, that some samples of protein and guanidine hydrochloride contain heavy metal impurities which can catalyze the oxidation of protein —SH groups by oxygen. In the present titrations, there-fore, the titrating agent is added before the protein is denatured by guanidine hydrochloride. Thus the denatured protein is not exposed to oxygen before it is exposed to the titrating agent.

To prevent oxidation of protein —SH groups by oxygen during the nitroprusside test in guanidine hydrochloride solution cyanide is added. The amount of cyanide needed to combine with the

[1] *p*-Chloromercuribenzoate has been used as an —SH reagent by Heller-man (Hellerman, L., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, 7, 165 (1939)).

[2] Greenstein, J. P., *J. Biol. Chem.*, 125, 501 (1938).

[3] Anson, M. L., *Science*, 90, 256 (1939).

[4] Anson, M. L., *J. Gen. Physiol.*, 23, 247 (1939).

heavy metal impurities is too small to cause any reduction of S—S to —SH. Cyanide must not be present, however, during the titrations with mercuribenzoate and ferricyanide, since cyanide interferes with the abolition of —SH groups by those reagents.

In agreement with previous results,[2-4] 1 cc. of 0.001 M titrating reagent is needed in the new titrations to abolish the —SH groups of 10 mg. of denatured egg albumin. The same value is obtained whether the titration is carried out in guanidine hydrochloride or duponol PC solution, whether the titrating agent is an oxidizing agent or a heavy metal compound, whether the abolition of the nitroprusside test or the failure to reduce ferricyanide is used as an end-point for the titration. The fact that very varied procedures yield the same titration value is strong evidence that only —SH groups are being titrated.

If the sample of protein or guanidine hydrochloride used happens to contain heavy metal impurities, then low results are obtained by the porphyrindin titration in its original form and there is no agreement with the results obtained by the present technique in which the titrating agent is added before denaturation.

The reactions of the —SH groups of denatured egg albumin in guanidine hydrochloride solution described in this note and also that of the —SH groups of native egg albumin with iodine described previously[3,5] can be applied to tobacco mosaic virus. The —SH groups of tobacco mosaic virus and the chemical and biological properties of tobacco mosaic virus which has reacted with iodine will be discussed in a later paper.

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[5] Anson, M. L., *J. Gen. Physiol.*, **23**, 321 (1940).

CONVECTION EFFECTS IN ELECTROPHORESIS

Sirs:

In electrophoretic studies, it is desirable to use as high a potential gradient as may be possible. At the same time it is often advantageous to use fairly concentrated buffers in order that boundary anomalies due to the Donnan equilibrium during dialysis and other similar conditions may be minimized.

The electrophoretic cell designed by Tiselius can dissipate heat rapidly, so that a fairly high current and a fairly high voltage do not produce enough heat to disturb the boundaries by convection. There are, however, few data as to the maximum power that may be used without disturbing the boundaries, although such information would be very useful.

In the course of an investigation not primarily designed to measure the optimum power, we have obtained some results that are of interest. The experiments were carried out in the Tiselius apparatus, with one cell section instead of the usual two. The water bath was kept at $0.45° \pm 0.05°$. A 0–150 milliammeter in series with the usual Ag-AgCl electrodes measured the current, and a high resistance voltmeter in parallel measured the voltage drop across the two electrodes. This, of course, is not identical with the drop across the cell, since the resistance of the buffer in the large electrode vessels is not small enough to be neglected. The Longsworth modification of the Toepler schlieren method was used to study the boundaries.

The boundaries studied were those due to a solution of 0.5 per cent sucrose in three buffers: (1) a buffer of pH 7.5, containing NaCl 0.15 M and $PO_4^{=}$ 0.02 M; (2) one of pH 7.3 containing NaCl 0.25 M, $PO_4^{=}$ 0.02 M; and (3) one of pH 6.9 containing K_2HPO_4-KH_2PO_4, $\mu = 0.2$ (0.1 M $PO_4^{=}$). The following conditions were observed: no current was passed through the system; current was passed and increased every hour; constant current was passed for several hours.

In this manner it was found that when no current was passed,

and thus no heat generated, the boundaries diffused normally and remained distinct for at least 20 hours.

When the current was increased hourly, the boundaries were disturbed, with the production of extra peaks only after the experiments had proceeded for about 5 hours and the product of current $\times$ voltage had exceeded 8. No differences that might be ascribed to the buffers were found, with the obvious exception that the voltage at which this figure was reached was highest for buffer (3) and lowest for buffer (2). In all cases in which two or more sharp bands were produced due to overheating, these bands disappeared and a single boundary was observed within 2 hours after the current was turned off and the cell allowed to cool.

For experiments at constant current, we chose to use $Ei = 6$, since this is considerably more than the power we ordinarily use (approximately 5 times more). In this case it was found that the experiment could proceed for at least 10 hours (the longest experiment made) without the formation of "convection" bands.

It would seem, then, that as far as freedom from convection disturbances is concerned, it is safe to use 8 watts for runs of 4 or 5 hours duration, and 6 watts for all ordinary purposes.

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THE ISOLATION OF Δ-5,7,9-ESTRATRIENOL-3-ONE-17 FROM THE URINE OF PREGNANT MARES*

Sirs:

From the non-phenolic extract of equine pregnancy urine there has been isolated a new hydroxy ketone which has been definitely characterized as a Δ-5,7,9-estratrienolone. Almost certainly the compound is the 3-hydroxy-17-keto derivative, a neutral isomer of estrone in which Ring B instead of Ring A is benzenoid.

Analyses of the hydroxy ketone (m.p. 138–139.5°, [α]$_D$ +59°), its acetate (m.p. 158°), and oxime (m.p. 195–197°) established the empirical formula, $C_{18}H_{22}O_2$, and the function of the 2 oxygen atoms. The presence of a benzenoid ring in the nucleus was indicated by a positive xanthoproteic reaction and a yellow color with tetranitromethane, and the absence of isolated double bonds, by saturation to bromine (Rosenmund-Kuhnhenn method; observed iodine number 5.2, theory for one ethylenic linkage 94). The ultraviolet absorption spectrum[1] exhibited maxima at 269.5 and 278 mμ, with ε = 345 and 240 respectively; these values are characteristic of Ring B aromatic steroids (*cf.* neoergostatriene[2] and epineoergosterol[3]). Proof of the Δ-5,7,9-estratriene skeleton and of the location of the substituents on C_3 and C_{17} was provided by the identification of the product of hydrogenation. 1 mole of hydrogen was taken up to yield a diol, $C_{18}H_{24}O_2$, with physical properties (m.p. 168°, [α]$_D$ −5°, m.p. of diacetate 115°) in good agreement with those of Δ-5,7,9-estratrienediol-3(β?),17(α).

[1] Our thanks are due Professor J. H. L. Johnstone and Mr. F. Warr for the determination.
[2] Haslewood, G. A. D., and Roe, E., *J. Chem. Soc.*, 465 (1935).
[3] Windaus, A., and Deppe, M., *Ber. chem. Ges.*, 70, 76 (1937).

The latter was obtained by Ruzicka *et al.*,[4] David,[5] and Marker *et al.*[6] on reduction of Ring A and the carbonyl group of equilenin (also from α-dihydroequilenin); Ruzicka *et al.* give m.p. 166.5°, m.p. of diacetate 115°, David, m.p. 166–168°, $[\alpha]_D$ −16°, m.p. of diacetate 117–118°, and Marker *et al.*, m.p. 172°. An authentic specimen of the diol diacetate from equilenin, kindly furnished by Professor L. Ruzicka, did not depress the melting point of that from the urinary hydroxy ketone.

Whether the compound is the 3-hydroxy-17-keto- or the 3-keto-17(α)-hydroxy-Δ-5,7,9-estratriene has not been established with certainty, as both could give rise to the above diol on hydrogenation. From biological considerations, the former derivative is much the more logical in view of the probable origin from equilenin by saturation of Ring A. This is substantiated by the magnitude of the decrease in dextrorotation observed on reduction of the ketone group to the alcohol, which is consistent with the change from a 17-keto to a 17(α)-hydroxy steroid, but considerably greater than the values pertaining to the conversion of 3-keto to 3(α or β)-hydroxy steroids. Also the behavior with the Zimmerman ketone reagents is more indicative of a 17- than a 3-keto compound. More conclusive evidence in this connection is being sought.

Full details will be published shortly.

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[4] Ruzicka, L., Müller, P., and Mörgeli, E., *Helv. chim. acta,* 21, 1394 (1938).

[5] David, K., *Acta brev. Neerl.,* 8, 211 (1938).

[6] Marker, R. E., Rohrmann, E., Wittle, E. L., and Tendick, F. H., *J. Am. Chem. Soc.,* 60, 2440 (1938).

DIFFERENTIATION OF RED BLOOD CELLS BY THEIR PSEUDOHEMOGLOBIN CONTENT

Sirs:

According to earlier work[1] the pseudohemoglobins (the source of the "easily split off" blood iron) accompany hemoglobin within the red blood cells and serve as intermediates in bilirubin formation. The hypothesis has been advanced[1,2] that the oxidative opening of the porphyrin ring (still combined with unaltered globin), a change which renders the iron easily detachable, is produced by nascent hydrogen peroxide. Since hydrogen peroxide is known to be a product of the oxidizing and reducing processes within the cells, this hypothesis involves the consequence that red cells of more advanced age should contain an increased percentage of pseudohemoglobin iron.

Such evidence as is available favors the assumption that decreased osmotic resistance might be a property of older erythrocytes. Hence a determination of both the hemoglobin and the pseudohemoglobin content of hemolysates obtained under varied hypotonic conditions from the red cells of a given sample of blood might yield evidence favoring or contradicting the idea mentioned above.

Human blood, freshly drawn and protected against coagulation by liquoid "Roche," was used as the source of the cells, which were washed several times with 0.9 per cent sodium chloride solution. A preliminary osmotic test was carried out to determine the minimal osmotic resistance. The most concentrated hypotonic solution was selected which could give a partial hemolysate of sufficient concentration to be tested for hemoglobin and pseudohemoglobin after removal of the unhemolyzed cells. Hemoglobin and pseudohemoglobin determinations were also performed on the

[1] Barkan, G., and Schales, O., *Z. physiol. Chem.*, **248**, 96 (1937). Barkan, G., *Klin. Woch.*, **16**, 1265 (1937). Barkan, G., and Walker, B. S., *J. Biol. Chem.*, **131**, 447 (1939).

[2] Barkan, G., and Schales, O., *Z. physiol. Chem.*, **253**, 83 (1938).

residual unhemolyzed cells, or on a sample of the whole blood cells, or on both. In one experiment we succeeded in continuing the partial hemolysis stepwise over a wider range of hypotonic solutions, thus fractionating the red cells still further.

The hemoglobin was determined by the Evelyn[3] photoelectric method. The hemoglobin iron was calculated on the basis of 0.336 per cent. For the determination of the pseudohemoglobin iron, our photoelectric method[4] was used.

Specimen No.	Blood cells	Hemolyzed with	Hb iron	Pseudohemoglobin iron	
			mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 mg. Hb iron*
1	Whole	0.45% NaCl (partial)	5.92	0.316	5.34
	"	Water (complete)	6.41	0.252	3.93
2	"	0.432% NaCl (partial)	0.574	0.023	4.01
	Residual*	Water (complete)	8.36	0.232	2.77
3	Whole	0.432% NaCl (partial)	0.872	0.047	5.39
	Residual*	Water (complete)	5.38	0.181	3.36
	Whole	" "	2.98	0.120	4.03
4	"	0.45% NaCl (partial)	0.20	0.0106	5.3
	Residual*	0.423% " "	1.02	0.036	3.53
	"	0.396% " "	6.72	0.24	3.57
	"	0.36% " "	16.4	0.532	3.24
	"	Water (complete)	6.97	0.209	3.00

* Residual cells from preceding hemolysis.

The table shows typical results. The percentage of pseudohemoglobin iron is, as a rule, higher in the osmotically less resistant cells. If, as assumed, the less resistant cells are the older ones, the result is significant; with no assumptions, it shows that red cells in a single blood sample vary in pseudohemoglobin content.

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[3] Evelyn, K. A., *J. Biol. Chem.*, **115**, 63 (1936).
[4] Barkan, G., and Walker, B. S., *J. Biol. Chem.*, **135**, 37 (1940).

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